THE INFLUENCE OF GAMMA RADIATION UPON SHELLSTOCK OYSTERS, AND CULTURABLE AND VIABLE BUT NONCULTURABLE *VIBRIO VULNIFICUS*

By

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The objectives of this research were to determine the effects of gamma radiation on Florida and Texas shellstock oysters in terms of shelf life and the microbial consequences. Oysters were evaluated for shell to meat ratios, shell dosimetry and surviving organism identification during dry, cold storage. The D_{10} values of log and stationary phase *Vibrio vulnificus* strains C7184 and mutant CVD 713 in ASW, as well as the VBNC form of each, were determined on a variety of media. Resuscitation after 24 and 48 hours at room temperature was monitored on solid media and compared to the corresponding direct viable count. True resuscitation was evaluated in terms of growout of a few remaining culturable cells, the effects of resuscitation media and the effect of different microbial concentrations.

The D_{20} values at 0.0, 1.0 and 3.0 kGy exposures for FL oysters were 17, 4 and 2 days, respectively, and 14, 4 and 2 days for TX shellstock oysters. The D_{50} values for FL shellstock oysters were >25, 9 and 3 days and the D_{50} values for TX shellstock oysters were >25, 11 and 4 days for the 0.0, 1.0 and 3.0 kGy exposures, respectively. Immediately following irradiation there is a decrease of about 2 logs in total bacterial numbers; however, the bacterial numbers return to the nonirradiated, control values. Some surviving organisms post-irradiation were identified as Vibrio spp., E. coli, Aeromonas, Pseudomonas and Serratia. The shell to meat ratios of FL, TX and LA oysters were 4.8, 5.5 and 7.2, respectively. Internal dosimeters recorded less dose inside the shell than the D_{max}/D_{min} ratios showed.

The D_{10} values for O, T and B were 0.057, 0.043, and 0.057 kGy, respectively, regardless of phase in artificial seawater. The D_{10} values of VBNC O, B, T = 0.165, 0.173 and 0.147 kGy, respectively. These D_{10} values were 3X larger than what was observed for the culturable cells. Resuscitation occurred after 48 hours, with levels matching the original direct viable count. Undiluted samples resuscitated back to the original DVC values, but none of the diluted samples resuscitated. Cell concentration played a significant role as concentrations below 10^5 per mL would not resuscitate.

INTRODUCTION

Since the 1980s, the shellfish industry has been the focus of a great deal of scrutiny because of the potential threat of illness associated with the consumption of raw oysters. The illness of concern is due to the Gram-negative, curved, rod-shaped bacteria Vibrio vulnificus, which are naturally occurring inhabitants of the estuarine and marine environments (Tamplin et al., 1982). The primary portals of entry into the human system are through the ingestion of the bacteria, which are located in the tissue of raw oysters and clams, or by exposure of wounds or skin lesions to seawater containing the organisms. Upon entry, Vibrio vulnificus leads to a rapidly developing primary septicemia, with accompanying hypotension, erythema and swelling of wounds and metastatic cutaneous lesions (Blake et al., 1979; Tacket et al., 1984). The bacteria are particularly dangerous and life-threatening to any individual who may suffer from a compromised immune system, cirrhosis, diabetes, AIDS, cancer, liver disease, or hemachromatosis, as these groups are at a higher risk for infection (Blake et al., 1979). It has been estimated that at least 45 million individuals in the U.S. are currently sensitive to foodborne disease, of which nearly

30 million are elderly people, 6 million are pregnant women, 4 million are neonates, and almost 3 million are cancer patients (CAST, 1994).

Vibrio vulnificus has a seasonal incidence in that it is found in the highest numbers in warm water (above 75°F) during the summer months, and it disappears in the cold waters of the winter months (Kelly, 1982; Tamplin et al., 1982). Furthermore, its presence is also dictated by the salinity of the seawater (Kelly, 1982; Tamplin et al., 1982). Thus, the presence of this microorganism in our food supply is of great concern and attempts to eliminate it from raw shellfish are being sought.

Since 1980, a tremendous amount of effort been put forth by shellfish regulatory agencies and the shellfish industry to reduce this public health concern. The efforts have tended to focus on alternative and novel methods to the current handling practices. The current, most accepted practice in the handling and storage of oysters is what is termed dry, cold storage. In this method, oysters are harvested, slightly cleaned, culled and either placed in croaker sacks or wax boxes and stored in a refrigerator at 34-36°F. This offers little in the way of decreasing bacterial counts and extending product shelf life. Hence, there are three primary methods of "cleaning up" oysters that have been researched most recently.

The three most widely investigated methods for cleaning up shellfish have been depuration, relaying and irradiation. Depuration is a method in which freshly harvested oysters are placed into large tanks of seawater that have been pre-equilibrated to about 70°F and to a similar salt concentration of the harvest area seawater. These oysters are then allowed to pump and purge clean seawater that is constantly being filtered, UV-sterilized and recirculated (Blogoslowski and Stewart, 1983; Rodrick and Schneider, 1991; Somerset, 1991; Jones et al., 1991). This method was originally thought of as the savior to the oyster industry; however, oysters do not completely purge themselves of all of the bacteria contained within their tissues (Rodrick et al., 1989), and some researchers actually report an increase in overall numbers of Vibrio post-depuration (Tamplin and Capers, 1992). Others report that Vibrios are readily depurated from Eastern oysters (Kelly and Dianuzzo, 1985; Groubert and Oliver, 1994). The same technique has been studied using ozone rather UV light as a sterilizer. Rodrick and Schneider (1991) examined the effects of an ozone generated oxidant concentration of 0.9 mg/L in a seawater tank at 23 ppt, pH = 8.2 and 19°C had on V. vulnificus cells, and found that after 60 minutes none of the 2500 CFU per 100 mL of seawater were detected. The study was repeated with cells artificially inoculated into clams,

and a 2-3 log decrease in numbers was observed (Rodrick and Schneider, 1991; Schneider et al., 1991).

Relaying is another method of transferring freshly harvested oysters to another seawater environment. However, in the case of relaying, oysters are moved from one harvest area to another approved harvest area and allowed to pump and purge contaminated seawater as filter feeders do. Eventually the oysters are harvested again and then ready for sale. As with depuration, this method has the serious limitation that not all of the Vibrio are shed and there are still some residual cells left in the oyster tissue (Richards, 1988). There is also a lack of approved waters for oysters to be transferred to, and there is virtually no control over environmental conditions. Furthermore, this method is quite labor intensive, and the oysters must remain in the approved waters for at least 15 days.

Perhaps the most effective method to eliminate V. vulnificus from shellstock oysters is through the use of ionizing radiation. This method is certain to kill all V. vulnificus present in oysters, provided enough dose is applied to achieve the desired number of D-value reductions. Vibrio vulnificus has been documented to be quite sensitive to low doses of irradiation (Dixon, 1992). However, there are other bacteria and viruses that have significantly higher D-values, and thus a higher dose may be required to eliminate them.

Furthermore, Dixon (1992) showed that winter oysters themselves are sensitive to irradiation and can be killed at doses of 2 kGy and up. Irradiation is a powerful processing aid; however, the doses necessary to ensure that all V. vulnificus are killed, as well as other Vibrios, other bacteria and viruses, are potentially lethal to oysters. This can have serious economic consequences to the processors, because of die-off (Dixon, 1992).

The specific research objectives were, first, to elucidate the influence that ionizing radiation has on a true commercial scale irradiation of oysters from Florida and Texas. The previous research in this area has been limited due to the irradiation of only a few bushels at a time. The influence of irradiation must be first evaluated in terms of the shellstock oyster shelf life over a two week period in dry, cold storage after irradiation. In terms of microbiological consequences, the total bacterial, total coliform, E. coli and V. vulnificus levels were determined in the oysters immediately following irradiation, as well as throughout dry, cold storage for 2 weeks after irradiation. The second objective was to assess the growback potential of any surviving Gram-negative organisms, by biochemically identifying the survivors. The third objective was to address the actual dose delivered to the oysters, as well as the amount of radiation that actually penetrated into

the interior of the shell of the oysters. Finally, a shell weight to meat weight ratio was calculated for FL and TX oysters post-irradiation, and on a separate occasion for LA oysters.

Other objectives of this research were to determine D_{10} values for the logarithmic and stationary phase of three forms of V. vulnificus, a.) opaque/virulent (O) C7184, b.) translucent/avirulent (T) C7184 and c.) an opaque/virulent/mutant (B) CVD 713 in artificial seawater (ASW) and plating on a variety of media. Previous research by Dixon (1992) determined D_{10} values for 24 hour cultures of V. vulnificus in phosphate buffered saline. This is a very limited approach as the natural environment for V. vulnificus is seawater, and a distinction between logarithmic and stationary phase cells should be made. Thus, the D_{10}'s of O's, T's and B's were determined for both log and stationary growth phase cells in ASW. Another objective was to determine the D_{10}'s for the viable but nonculturable (VBNC) state of O, T and B in ASW at 4°C. The VBNC cells were assessed post-irradiation in terms of a direct viable count (DVC) and total cell count post-irradiation (AODC). The remaining objectives were evaluating the ability of VBNC cells to resuscitate post-irradiation on five different media and examining the effect of factors such as dilution of samples prior to resuscitation and the use of fresh resuscitation media.

REVIEW OF THE LITERATURE

Vibrio vulnificus

There exists a public health risk for certain high-risk individuals who consume raw or inadequately cooked oysters or clams, or who expose wounds or skin lesions to contaminated seawater. The American oyster, Crassostrea virginica, and the hard-shelled clam, Mercenaria campechiensis, have been implicated many times as the source of serious foodborne, enteric illness outbreaks (Blake et al., 1980; Blake, 1983; DuPont, 1986). Many different bacterial and viral agents have been isolated from such shellfish including the Vibrio, Salmonella and Shigella species, Hepatitis and Norwalk viruses (Blake et al., 1980).

Vibrio vulnificus Characteristics

Vibrio vulnificus is a Gram-negative, halophilic, rod-shaped bacterium that is native to many estuarine or marine environments (Blake, 1983; Dupont, 1986). In 1964, the Special Bacteriological Section of the Centers for Disease Control identified Vibrio vulnificus as a lactose-fermenting variant of V. parahaemolyticus (Blake et al., 1980). Many different lactose-positive Vibrio strains were studied by Baumann et al. (1973) and they subsequently named them Beneckea vulnifica. In 1976, the

Center for Disease Control officially referred to this organism as a lactose-positive Vibrio (Hollis et al., 1976). Finally in 1980, Farmer proposed that B. vulnifica be classified as V. vulnificus (Farmer, 1979; Farmer, 1980).

Vibrio vulnificus continues to be the focus of a great deal of research because of its unusual characteristics. Vibrio vulnificus is a highly invasive organism (Morris and Black, 1985) and it is known to produce a variety of toxic or virulence factors such as cytolysin, enterotoxin, protease, phospholipase A and siderophores (Kreger and Lockwood, 1981; Stelma, et al., 1988; Gray and Kreger, 1985; Shinoda et al., 1985; Kothary and Kreger, 1985; Smith and Merkel, 1982; Testa et al., 1984; Simpson and Oliver, 1983). The cytolysin has been shown to possess vascular permeability enhancing activity. The proteases are of particular interest since they have been shown to enhance vascular permeability in rat skin due to the release of histamine from mast cells, as well as activating the plasma kallikrein-kinin system, leading to bradykinin production, which enhances membrane permeability (Miyoshi et al., 1986; Miyoshi et al., 1987).

Virulent (O) Versus Avirulent (T)

Two distinct colony morphotypes exist in V. vulnificus, with one being the opaque (O) or encapsulated, and the other being the translucent (T) or

nonencapsulated. The virulent, encapsulated form of V. vulnificus is of greater concern than the avirulent, nonencapsulated form (Amako et al., 1984; Tsuru et al., 1987). Another form of V. vulnificus also exists and it has been referred to as the viable but non-culturable (VBNC) state (Colwell, 1984; Birbari and Rodrick, 1990). In this form, the bacterium is not able to be detected by standard microbiological plating procedures. Growth of VBNC V. vulnificus is not present in pour plates, spread plates and broths, yet VBNC V. vulnificus is a viable, metabolically active cell. Instead, this form must be detected by alternative means such as by fluorescent antibody testing (Bryton and Colwell, 1987), or by the yeast extract-nalidixic acid incubation method (Kogure et al., 1979).

The capsule of V. vulnificus was determined to be an acidic polysaccharide and accounts for the colony opacity and virulence (Kreger et al., 1984). Amako et al. (1984) further substantiated the difference between O's and T's when electron microscopy revealed that T's had less polysaccharide present than the O's. Yoshida et al. (1985) performed some studies on colony opacity in V. vulnificus and found that serum resistance, tissue invasiveness, antiphagocytic activity and cell lethality were positively correlated with colony opacity. Antiphagocytic activity was also previously documented by Kreger et al. (1981). The structure of the capsular

polysaccharide was determined by Reddy et al. (1992) in strain M06-24 by NMR spectroscopy. The polymer is composed of a repeating structure of four sugar residues per repeating subunit: three residues of 2-acetamido-2,6-dodeoxyhexopyranose in the α-gluco configuration (QuiNAc) and an additional residue of 2-acetamido hexouronate in the α-galactopyranose configuration (GalNAcA) (Reddy et al., 1992). This polymer was also produced in the equivalent T morphotype cells (Reddy et al., 1992).

The T morphotype is not just simply avirulent, but instead they are perhaps less virulent. These cells have the ability to spontaneously change back and forth between the O morphotype and the T morphotype. Identical V. vulnificus strains (E4125) were tested by Simpson et al. (1987) and Stelma et al. (1988). Simpson et al. (1987) found the strain to be lethal to mice, whereas Stelma et al. (1988) did not. These data tend to indicate the conversion between morphotypes within the same strain. There is also controversy surrounding this type of morphotype conversion in that only O to T conversion exists (Simpson et al., 1987), whereas a two-way exchange of morphotypes has been reported in strain MO6-24 (Wright et al., 1990). The frequency of isolation of T's from an MO6-24 O culture was 2.2×10^{-4}, and the T

colonies reverted to the O morphology at a frequency of 9.2×10^{-3} (Wright et al., 1990).

Three clinical strains and one environmental strain of Vibrio vulnificus were studied by Tsuru et al. (1987), and it was determined that addition of 2.5% NaCl to growth medium resulted in a loss of capsular material, and a subsequent decrease in virulence. In addition, growth in 0.5% NaCl results in an increase in capsular material, and thus increased virulence (Tsuru et al., 1987).

The amount of bacteria necessary to cause disease is often referred to as the lethal dose 50 (LD_{50}). An LD_{50} for four strains of V. vulnificus was found to be 4.7, 5.7, 6.2 and 7.3 $\log_{10}$ for O's and 7.9, 8.3, 8.8, and 8.5 $\log_{10}$ for T's when injected intravenously into mice (Yoshida et al., 1985). The LD_{50} was determined to be 4×10^6 and 1.9×10^8 for strain C7184 O's and T's, respectively, when injected intraperitoneally in mice (Simspon et al., 1987). This LD_{50} was significantly lower when V. vulnificus was injected subcutaneously in liver damaged or iron overloaded mice. The LD_{50} was reduced to 1×10^2 and 1×10^3 for the O's and T's, respectively (Brennaman et al., 1987). Strain MO6-24 was also tested in iron overloaded mice and LD50's were found to be $<10^2$ and 3×10^5 for the O's and T's. Only opaque colonies were detected and isolated after passage through the mouse (Wright et al., 1990).

This type of testing continued with 24 clinical and environmental isolates of V. vulnificus being injected into iron-overloaded and cyclophosphamide treated mice. The result was that 17 of the 24 showed a ≥ 3.5 $\log_{10}$ decrease in the LD_{50} (Stelma et al., 1992). Furthermore, four out of the seven strains that were classified as avirulent initially turned out to be virulent after being injected into iron overloaded and immunosuppressed mice, while the remaining three staying avirulent (Stelma et al., 1992). Finally, no siginificant difference was detected between the environmental and clinical strains (Stelma et al., 1992).

Ecology and Epidemiology

The organism V. vulnificus exists most commonly along the U.S. Gulf Coast (Tamplin et al., 1982; Kelly, 1982), but is also found along the Atlantic Coast (Oliver et al., 1983; O'Neill, 1990) and the Pacific Coast (Kelly and Stroh, 1988; Kaysner et al., 1987). Salinity and water temperature play a role in the detection of the organism, as the numbers of V. vulnificus are much higher in warmer waters and but much lower in salinities of greater than 35 and 38 ppt (Tamplin et al., 1982; Kelly, 1982; Tamplin, 1990; Kaspar and Tamplin, 1993).

Vibrio vulnificus is a ubiquitous marine and estuarine microorganism that has been found throughout the world. Multiple strains of the organism have been

isolated throughout the marine environment, including seawater, sediment, plankton and a variety of sealife including oysters and clams (Tamplin et al., 1982; Tamplin et al., 1991; Buchreiser et al., 1995). Its existence in the environment is not related to fecal pollution, and thus is considered naturally occurring (Tamplin et al., 1982; Rodrick et al., 1994). This finding was corroborated by Koh et al. (1994) who found either negative or no correlation between Vibrios and E. coli, enterococci, fecal coliforms and total coliforms in Apalachicola, Bay, FL seawater.

It has been reported by Bonner et al. (1983) that there were 23 cases of V. vulnificus reported between July 1972 and June 1982. Klontz et al. (1988) studied 62 cases of V. vulnificus infections in Florida between 1981 and 1987. Forty-five of these cases were due to consumption of shellfish, with the other 17 due to wound infections from contaminated seawater. Fifteen of these patients died and all had underlying conditions such as liver disease (Klontz et al., 1988). Howard and Lieb (1988) reported that 51 patients with soft tissue infections due to Vibrios were reported between October 1, 1981 and November 30, 1986 in Florida. Thirteen of these patients died, with 11 directly attributed to V. vulnificus (Howard and Lieb, 1988). The U.S. Food and Drug Administration reported that there were 60 reported

cases of V. vulnificus infections between 1975 and 1988, and 36 of these cases ended in death (FDA, 1989).

Klontz et al. (1993) reported that 37 cases of Vibrio infections occurred in Florida between January 1989 and December, 1990, and 89% (33) had gastroenteritis, 3% (1) septicemia, 5% (2) had wound infections and the other patient yielded insufficient data for characterization. Excluding the wound patients, 33 out of 34 patients reported that they had consumed raw oysters the week before the onset of illness. The Vibrio species isolated included V. parahaemolyticus (9 patients), V. cholerae non-01 (7 patients), V. hollisae (5 patients), V. vulnificus (5 patients), V. fluvialis (4 patients), V. mimicus (4 patients) and V. cholerae 01 (1 patient). No deaths resulted.

There were 138 cases of V. vulnificus infections reported between 1988 and 1993 across the U.S. Gulf Coast. The breakdown among states was Florida = 57, Alabama = 21, Louisiana = 34, Mississippi = 3, and Texas = 23. Among these 138 cases, there were 45 deaths for an overall mortality rate of 42%, and a range of 0% in MS to 57% in Texas (CDC, 1993).

The most recent report of V. vulnificus deaths is a 1995 report by the Center for Disease Control (CDC) stating that as of October 1, 1995, there were 23 cases in Florida resulting in 10 deaths. Sixteen of the cases were traced back to shellfish consumption, with four

deaths directly attributed to the consumption of clams harvested from Indian River, FL (Food and Chemical News, 10/23/95).

Vibrio strains are found throughout the world and isolation investigations have been made. were investigated for there toxigenic characteristics. Approximately 33% of the Vibrio strains isolated from food available in Taiwan showed hemolytic activity, while almost one-half showed either cytotonic or cytotoxic activity, with 20% or more showing heat-stable cytotonic or cytotoxic activity. Finally, about 70% of the Vibrios isolated showed mouse lethality (Wong et al., 1993).

Vibrio Vulnificus Infections

As mentioned, infection by V. vulnificus arises from the ingestion of raw or inadequately cooked oysters or clams or by the exposure of skin lesions or wounds to contaminated seawater. After ingestion of V. vulnificus, a primary septicemia results that is accompanied by gastroenteritis, chills, fever and prostration. In contrast, those individuals who become infected with V. vulnificus through a preexisting skin abrasion exhibit symptoms such as rapid swelling and erythema around the lesion, fever and chills (Blake et al., 1980; Rodrick et al., 1982). Wound infections can also lead to myositis and severe cellulitis and furthermore, a likeness to gas gangrene (Klontz et al., 1988).

Vibrio vulnificus infections are rapidly induced with median incubation periods of approximately 12-16 hours (Blake et al., 1980). Vibrio vulnificus infections can also be life-threatening in that approximately 50% of patients developing primary septicemia die (Morris and Black, 1985). This mortality rate can reach 90% in those patients developing hypotension within 12 hours after hospital admission (Klontz et al., 1988). Once primary septicemia sets in, most patients begin to develop secondary lesions on their extremities that can result in a necrotizing vasculitis in the muscles, which may often result in amputations (Howard et al., 1986).

Several epidemiological studies have been conducted and the results indicate a relationship between primary septicemia and several pre-existing conditions in the patients. Some of these conditions include cirrhosis, diabetes, hemochromatosis, kidney failure, liver and iron disorders, and any other immunocompromising conditions (Blake et al., 1979; Tacket et al., 1984; Johnston et al., 1985). It has also been shown that the virulence of V. vulnificus is associated with a cellular capsule that is composed of acidic polysaccharide (Amako et al., 1984; Yoshida et al., 1985). Furthermore, iron appears to be the essential element in the role of pathogenicity as patients with cirrhosis and hemochromatosis can have elevated serum iron levels and it is these patients that are at greatest risk for infection (Wright et al., 1981;

Howard and Lieb, 1986). In addition, mice injected with V. vulnificus develop severe edema fluid accumulation leading to extreme hemoconcentration. Thus, this accumulation is quite important in the pathogenesis of V. vulnificus (Poole and Oliver, 1978; Bowdre et al., 1981).

Viable But Nonculturable Vibrio Vulnificus

Considering that the majority of V. vulnificus infections occur during the warm summer months, especially when the water has been above 25°C for many months, the question that arises is what happens to the cells during the winter. It was once assumed that when water temperatures reached a low enough level, the V. vulnificus cells were dying in the shellfish and in the seawater. This was assumed because recovery of V. vulnificus cells from shellfish and seawater was not possible during the cold winter months. However, more recent studies have shown that V. vulnificus may be entering a "viable but nonculturable" (VBNC) physiological state. The exact mechanism for this transformation has not been clearly elucidated; however, this may be a response to sublethal injury or may be a survival strategy employed by V. vulnificus to live through dangerous environmental stresses such as low temperature, depleted nutrients or increased salinity. It is well known that bacteria will undergo size reduction to maximize surface to volume ratio in nutrient depleted environments (Morita, 1988). This VBNC state

has been documented by several researchers, and it is of particular concern in that these cells will not grow on conventional microbiological media (agars and broths), yet they remain viable (Colwell, 1988; Oliver et al., 1991).

There has been a great deal of conflicting data reported surrounding the viability and culturability of V. vulnificus at reduced temperatures. Some reports claim that Vibrionaceae grow at refrigerated temperatures (Hood and Ness, 1984), while others claim that there is a rapid loss in culturability in cells stored below approximately 10°C (Oliver and Wanucha, 1989; Weichart et al., 1992).

Linder and Oliver (1989) performed some of the pioneering work in the area of VBNC V. vulnificus. Their work showed that V. vulnificus cells, when added to artificial seawater microcosms at 5°C, became nonculturable after 24 days, and remained metabolically active for 32 days. Furthermore, once the cells became nonculturable on selective and nonselective media, the major fatty acid species (C_{16} and $C_{16:1}$) had dropped 57% from the original level, and there was the appearance of several short chain fatty acids and novel proteins (Oliver and Stringer, 1984; Hood et al., 1986; Linder and Oliver, 1989). These new products are an indication of the cells trying to survive the cold temperature and lack of nutrients. Finally, nonculturable V. vulnificus cells

were rounded, reduced in size, had fewer ribosomes and a loss of virulence in mouse infectivity studies (Linder and Oliver, 1989). There was then a great deal of question whether or not the formation of VBNC V. vulnificus cells were related to the bacterial starvation response.

As far as other bacteria are concerned, the VBNC state has been documented to exist for a variety of genera. Besides V. vulnificus, the VBNC state has been documented in Salmonella enteritidis (Roszak et al., 1984), V. cholerae (Colwell et al., 1985), and Campylobacter jejuni (Rollins and Colwell, 1986). Gram-positive bacteria such as Streptococcus faecalis, Micrococcus flavus and Bacillus subtilis have also been determined to experience a decrease in culturability in drinking water (Byrd et al., 1991).

Nutrient Starvation

It has been well known for many years that bacteria undergo a reduction in size as a means for survival in adverse environmental conditions such as depleted nutrients (Roszak and Colwell, 1987b). This starvation response has been studied using the marine Vibrio ANT-300 and comparing its starvation response to that of V. cholerae (Novitsky and Morita, 1976; Novitsky and Morita, 1977; Novitsky and Morita, 1978; Amy et al., 1983a, b; Baker et al., 1983; Hood et al., 1986). The two organisms responded similarly in that both lost their

intracellular granules, experienced a compacting of the nuclear region, a rapid decrease in cellular integrity and a conservation of ribosomal structures (Novitsky and Morita, 1976; Amy et al., 1983a, b; Baker et al., 1983; Hood et al., 1986). Vibrio cholerae showed convolution of the cell wall, while the ANT-300 showed little distortion. Hood et al. (1986) explained the difference in these organisms was due to V. cholerae coming from estuaries where there was always a flux nutrients, versus ANT-300 which, when living in open water, is constantly under depleted nutrient conditions. Subsequent studies on Vibrio spp. have shown that there are genes located on the chromosome that are responsible for size reduction (Smigielski et al., 1990). ANT-300 has also been shown to gain increased heat tolerance after starvation at 17°C (Preyer and Oliver, 1993).

Starvation, Temperature and VBNC

The three main factors affecting entry into the VBNC state are temperature, starvation or both. Oliver and Wanucha (1989) showed that at 15°C, V. vulnificus is capable of significant growth with excess available nutrients. However, a rapid decrease in culturability was observed at 5°C and 10°C even in the presence of excess available nutrients. This loss in culturability usually came within five days, yet direct viable counts indicated a large number of remaining viable cells (Oliver and Wanucha, 1989). Furthermore, an amino acid

uptake study was performed, and it was demonstrated that V. vulnificus would respire at 9°C and 13°C, but not grow (Oliver and Wanucha, 1989). Birbari and Rodrick (1991) and Wolf and Oliver (1992) showed that entry into the VBNC form of V. vulnificus was temperature dependent, as VBNC cells of V. vulnificus could be induced in ASW microcosms at 4°C after 21-35 days and 40 days at 5°C.

Oliver et al. (1991) analyzed 10 factors that may affect entry into the VBNC state and found that only the physiological age of the cells severely affected the time it takes for cells to become nonculturable, as stationary phase cells took longer than log-phase cells to be induced. Oliver et al. (1991) also showed that if cells were starved for 24 hours at room temperature and then incubated at 5°C, the cells remained culturable. This finding implies that there were some starvation proteins synthesized that actually repressed the VBNC state (Oliver et al., 1991). Starvation protein synthesis has been demonstrated by Amy and Morita (1983) in other marine bacteria. Morton and Oliver (1995) identified 34 carbon starvation-induced proteins in V. vulnificus. These proteins were induced over the 26 hours of carbon starvation, however it was determined that the proteins required for starvation survival are made within the first four hours of starvation (Morton and Oliver, 1995). Furthermore, V. vulnificus has been shown to induce 40 cold-responsive proteins to temperature downshifts from

23°C to 13°C as well as an overall diminution of protein synthesis and a new growth rate (McGovern and Oliver, 1995).

Resuscitation

The VBNC state of the organism also has the ability to resuscitate to its original culturable form. When a sample of VBNC cells is taken from an ASW microcosm at 5°C, and placed at room temperature for about 2-3 days, the cell counts return to the original bacterial numbers inoculated to start the microcosms (Nilsson et al., 1991). No increase in the total cell count was observed during resuscitation, thus indicating that the numbers observed on plate counts were not due to the growth of a few culturable cells (Nilsson et al., 1991). Furthermore, the cells had changed from small cocci of 1 μm to 3 μm rods. Oliver and Bockian (1995) showed that when VBNC V. vulnificus cells were injected into mice, resuscitation and subsequent infection occurred. This indicates that VBNC cells retain their virulence at least for some time after losing culturability (Oliver and Bockian, 1995).

Perhaps an even greater finding has to do with V. vulnificus resuscitation in the environment. Oliver et al. (1995) showed that when VBNC cells of V. vulnificus were placed into estuarine seawater at 15°C in January and February, the result was a complete resuscitation to the fully culturable state regardless of morphotype or

growth (log or stationary) phase. This study indicates that V. vulnificus may in fact enter into the VBNC state in the environment and this may be why it goes undetectable in the cold winter months (Oliver et al., 1995).

However, the resuscitation of VBNC bacteria is questioned by researchers, who instead explain the phenomenon as the growth and proliferation of a few surviving culturable cells. Ravel et al. (1995) attempted to distinguish between true temperature upshift resuscitation, and apparent resuscitation by the regrowth of a few V. cholerae cells that remained culturable. These workers diluted samples of VBNC cells 10- and 100-fold. Thus, if true resuscitation occurred, these two samples should have 10- and 100-fold less counts than the nondiluted control. Similar counts were observed in undiluted and 10- and 100-fold diluted samples indicating that V. cholerae is capable of growth in ASW. However, it could not be ruled out that a few cells underwent resuscitation, followed by subsequent growth (Ravel et al., 1995). Such regrowth has not been shown with V. vulnificus.

Detection Methodology

The viability of VBNC cells can be easily detected by indirect procedures such as fluorescent antibody staining (Bryton and Colwell, 1987), INT-formazan (Zimmerman, et al., 1978), and by the nalidixic acid-

yeast extract method (Direct Viable Count) (Kogure et al., 1979). It is the nalidixic acid-yeast extract method that will be used in this work because of its relative ease and accuracy. Yeast extract is used to stimulate cell growth, however cell division is inhibited in Gram-negative bacteria due to the presence of nalidixic acid. This DVC method was tested against a modified DVC method which incorporated radiolabelled substrates in microautoradiographic analysis to assess bacterial survival in laboratory microcosms. No significant difference was detected between the DVC enumerations with or without added radiolabelled substrate, thus the DVC was determined to be effective for enumerating the number of metabolically active bacteria in an aquatic sample (Roszak and Colwell, 1987a). Other methods such as polymerase chain reaction (PCR) can also be used (Brauns et al., 1991; Hill et al., 1991), but any DNA that may be present in a sample (dead cells or contamination) would be amplified thus altering the results. Furthermore, VBNC cells have been shown to have thicker cell walls that are resistant to the sonication used in PCR, and may have undergone DNA rearrangement after changing to the VBNC state. Researchers have also shown that these cells can be resuscitated to a culturable form after warming up the cells in artificial seawater to room temperature. The fact that these cells can recover from environmental

stresses and proliferate is of great concern because the capacity to cause disease is still potentially possible (Brauns et al., 1991).

Vibrio Vulnificus in Oysters

Hood et al. (1983) showed that the numbers of Vibrio spp. in shellstock Crassostrea virginica and Mercenaria campechiensis increased when stored at 2°C or 8°C. Furthermore, total counts of bacteria, fungi, coliforms, fecal streptococci, Aeromonas hydrophila and Clostridium spp. were significantly higher in shucked meats compared to those stored as shellstock. Similarly, Kaysner et al. (1989) found that V. vulnificus in shellstock and shucked oysters survived and multiplied under refrigeration. Conversely, Cook and Ruple (1989) and Ruple et al. (1989) found that Vibrionaceae do not multiply in shellstock oysters during dry storage at 10°C. This finding was corroborated by Murphy and Oliver (1992) who found that the number of O's and T's declined over a ten day storage time at 0.5 and 5.0°C. Kaspar and Tamplin (1993) showed that seawater salinities of 30, 35, and 38 ppt reduced V. vulnificus levels 58%, 88%, and 83%, respectively. In addition, the numbers of V. vulnificus in shellstock oysters were reduced 10- and 100-fold after 14 days at 2°C to 4°C and 0°C, respectively. Cook (1994) showed that at below 13°C, V. vulnificus failed to multiply in shellstock oysters. However, at temperatures of 18°C for 30 hours and ambient air temperature for 12 and 30 hours,

V. vulnificus levels were statistically higher ($p < 0.05$) than the oysters immediately after harvest.

The effects of frozen storage and vacuum-packaging on the survival of *V. vulnificus* in Gulf Coast oysters (*Crassostrea virginica*) was analyzed by Parker et al. (1994) and it was determined that 7-70 days of frozen storage led to a 3.5 to 4.0 $\log_{10}$ reduction in the number of total aerobic bacteria and *V. vulnificus*. Also, vacuum-packaged samples had significantly lower concentrations of *V. vulnificus* over the length of the study than did the normally packaged samples (Parker et al., 1994). This is not a very practical approach for the raw market; however this does have merit for the shucked meat products.

The effects of some chemical food additives on natural *V. vulnificus* populations in shucked oysters was evaluated. Sodium lactate (1 and 2%) and Fish-Gard (0.25 and 0.50%) were added to shucked oysters, and a significant difference in "*Vibrio*-like" counts was detected in the Fish-Gard treatments. No significant difference was detected in the total bacterial counts or drip loss (Darling, 1993). It was also determined that diacetyl at 0.05% concentration or greater could significantly reduce the natural numbers of *V. vulnificus* present in oysters, whereas lactic acid and BHA were not effective at concentrations up to 0.05% (Sun and Oliver, 1994). Two other interesting food ingredients to be

tested were Tabasco® sauce and horse-radish based cocktail sauce. These compounds were placed on freshly shucked oysters and incubated for ten minutes on the half shell. Results indicate that only the Tabasco® sauce was highly effective in reducing meat surface V. vulnificus and that little or no reduction was observed within the oyster tissue itself, regardless of which sauce was used (Sun and Oliver, 1995). The only components of Tabasco® are peppers, vinegar and water, and it is unclear whether it is these elements or the low pH that results in the bacterial reduction (Sun and Oliver, 1995).

One fact that is often overlooked is that V. vulnificus is in reality, an unwelcome guest in the oyster or clam. When V. vulnificus is taken up by oysters, it becomes the subject of phagocytic activity by clam hemocytes (Ulrich, 1982). Ulrich (1982) showed that granulocytes were the dominant hemocyte type interacting with V. vulnificus and Bacillus megaterium in the clam, Mercenaria campechiensis, whereas fibrocytes and hyalinocytes showed a lesser degree of activity. Furthermore, as temperature increases, phagocytic activity increases (Ulrich, 1982). The association of V. vulnificus with hemocytes increases with time, temperature and the initial V. vulnificus cell number / hemocyte number ratio (Harris-Young et al., 1993). Pretreatment of V. vulnificus with serum or an increased serum concentration did not increase V. vulnificus-

hemocyte associations, suggesting the absence of opsonic activity (Harris-Young et al., 1993). Another key finding of this study was that only 10-20% of the hemocytes bound the O morphotype versus approximately 50% for the T morphotype, indicating that encapsulation does have antiphagocytic activity (Harris-Young et al., 1993). This study was followed up with a comparison of the level of phagocytosis and bacterial degradation experienced by O and T V. vulnificus, V. cholerae and E. coli. It was determined that the levels of phagocytosis of O V. vulnificus was less than that of the T morphotype. The rate of intracellular death of O V. vulnificus was far less than the intracellular death rate experienced by E. coli or V. cholerae, even though the ingestion and uptake rates were similar. Finally, there was no significant difference observed in the levels of lysozyme and acid phosphatase activity in hemocyte monolayers inoculated with V. vulnificus (Harris-Young et al., 1995).

Radiation

The term radiation covers a wide range of different energies and can be broken up into two main forms, ionizing and nonionizing. Nonionizing radiation contains lower energy and include radiowaves, infrared, microwaves and visible light. Ionizing radiation consists of higher energy and includes primarily gamma rays and X-rays.

Radiation by definition, refers to an energy transfer from one material to another material. This

energy transfer may result in the material being heated, as with microwave radiation. If the energy level of the radiation is high enough, an electron may be removed from an atom (ionization). Upon ionization, an orbital electron is removed from an atom (excited electron), thus producing a free radical or a positively or negatively charged ion. A definitive amount of energy is required to induce such an ionization and thus to excite the electron. Once that amount of energy is absorbed by the material, the excited electron can become free of the nucleus (Urbain, 1986). Once free, the excited electron can now interact with other nearby materials and thus lead to an alteration in the chemical structure of the material. This alteration in the chemical structure of a material leads to the damage experienced by any biological flora present in the product (Elias and Cohen, 1983).

Radiation can also be divided into electromagnetic and particulate (corpuscular) radiation. Electromagnetic radiation is composed of self-propagating electric and magnetic disturbances, such as gamma rays and X-rays, characterized by specific wavelengths and frequencies (Urbain, 1986). Gamma rays emitted by the radioisotopes Cobalt-60 or Cesium-137 are the primary sources radiation used in food irradiation processing. Gamma rays are excess energy emitted from the unstable, radioactive nucleus of Cobalt-60 as it decays to its stable,

nonradioactive form of Cobalt-59. Particulate radiation is different from electromagnetic radiation as it is dependent upon the mass of a particle and its main constituents are the alpha and beta rays. These particles are set into motion by the use of an accelerator, which results in the production of kinetic energy (Urbain, 1986). These moving particles can then be aimed and directed at a product for exposure. This type of radiation consists of alpha particles, electrons etc.

Historical Perspectives of Food Irradiation

Food irradiation is a process where food products are exposed to specific doses of ionizing radiation to eliminate or sterilize insects, pests and bacteria. The result expected is a product that has an extended shelf life, and an enhanced product safety. Food irradiation, however, is by no means a new processing technique. X-rays were discovered in 1895 by Roentgen and radioactivity was discovered by Becquerel in 1896 (Josephson and Peterson, 1982). In the early 1900's, irradiation was tested on many different products, as in 1916, where irradiation processing of strawberries was being conducted in Sweden (Webb et al., 1987). The United States was also involved in 1921, when the first U.S. patent was submitted by Schwartz to use X-rays in eliminating Trichinella spiralis from pork. Subsequently in 1930, France had its first patent submitted by Wust,

who wanted to irradiate all foods (Josephson and Peterson, 1982). However, significant progress in the field of food irradiation was slow in developing and it was not until the early 1950's that any major research in food irradiation was performed.

In 1953, President Dwight Eisenhower unveiled his "Atoms For Peace Program" which called for the investigation of nuclear technologies (Webb et al., 1987; Josephson and Peterson, 1982). At this time, the U.S. Army (Natick Labs in Massachusetts) began the first extensive research in food irradiation for use on army field rations (Josephson and Peterson, 1982). It was many more years before the U.S. jumped into the irradiation arena. In 1957, the first commercial use of food irradiation was undertaken in Germany, to sterilize spices used in sausage making. In 1958, the German government repealed its approval. Also in 1958, the Soviet Union approved potato irradiation to prevent sprouting and in 1959, grain irradiation was approved for insect disinfestation (Webb et al.,1987).

Part of the reason that the U.S. has lagged behind other countries in the development of food irradiation technologies can be explained by the indoctrination of the United States' Food, Drug and Cosmetic Act's Food Additives Amendment of 1958. This amendment tabbed food irradiation as a food additive, not a process, and thus its use must be approved by petition similar to other

additives like microbial inhibitors or food colorings. Furthermore, food safety, nutritional quality and wholesomeness testing was required for approval. In 1963, the USFDA issued its first irradiation permit for bacon, packed in tin cans, at 4.5 Mrad. After bacon, many other products gained approval, such as wheat irradiation at 50,000 rads for disinfestation in 1963 and potato irradiation at 10,000 rads to inhibit sprouting in 1964 (Josephson and Peterson, 1982). Five years later, the FDA withdrew its permit for bacon irradiation, claiming that there were adverse effects in the bacon and that the safety testing had been poorly performed (Webb et al., 1987).

After rescinding bacon's approval, other products were very slow to gain approval in the U.S. In 1983, FDA approved herbs, spices and vegetable seasonings for irradiation for killing/sterilizing insects and controlling microorganisms. In 1985, FDA approved fresh pork irradiation to control Trichinellae, as well as the irradiation of dry enzyme preparations used in fermentation type processes to control insects and microorganisms. In 1986, the trend continued with fresh fruits, vegetables and grains gaining approval to control insects and delay ripening (Webb, et al., 1987). Chicken has been most recently approved for irradiation to control the levels of Salmonella in fresh and frozen poultry (C.F.R., Title 21, Part 179, 4-1-94). Several

recent food additive petitions have been filed and are still pending with the FDA. One is by United States Harvest Technologies for the irradiation of shellfish at 1.0 and 3.0 kGy. The other petition concerns the irradiation of fresh red meat (beef), primarily to control *E. coli* 0157:H7. Though there are a variety of products that are approved for irradiation, the US food industry is not irradiating foods on any large scale (IAEA, 1989). Table 1. shows the current regulatory status of food irradiation, as approved by the Food and Drug Administration. These products listed can be legally irradiated without petition, however the accompanying labelling must be affixed to the product so that the consumer may have proper notification of what treatment has actually been applied to the food (CFR, Title 21, Part 179, 4-1-1994).

Table 1. Food products that can be legally irradiated in the U.S., and approved doses.

APPROVED FOOD USE	DOSE REQUIREMENTS
For control of *Trichinella spiralis* in pork	Minimum = 0.3 kGy Maximum = 1.0 kGy
For growth and maturation inhibition of fresh foods	Maximum = 1.0 kGy
For disinfestation of arthropod pests in food	Maximum = 1.0 kGy
For microbial disinfection of dry or dehydrated enzyme preparations	Maximum = 10.0 kGy
For microbial disinfection of certain aromatic vegetable substances used solely as flavorings	Maximum = 30.0 kGy
For control of food-borne pathogens in fresh or frozen uncooked poultry	Maximum = 3 kGy (may not exclude O_2)

Food Irradiation Processing

Radiation Source Selection

Typically, the FDA approved sources of ionizing radiation used in food irradiation are the gamma ray emission from the radioisotopes, Cobalt-60 and Cesium-137. Cobalt-60 emits two gamma rays during decay, 1.17 and 1.33 million electron volts (MeV) each, whereas Cesium-137 emits a single 0.66 MeV gamma ray during decay. The halflife of Cobalt-60 is 5.3 years, while the halflife of Cesium-137 is about six times longer at 30.2 years. It should be noted that energy levels greater than 15 MeV are required to induce significant radioactivity in an irradiated food. Thus, Cobalt-60 (1.17 and 1.33 MeV) and Cesium-137 (0.66 MeV) are well below the 15 MeV necessary to induce radioactivity and thus there use in a food irradiation process will not result in induced radioactivity in the food (Urbain, 1986). Gamma rays are highly recommended in food irradiation because they offer maximum penetration into the food matter. The main disadvantage is that they are generated by a constant source (the radioactive element Cobalt-60) and thus there is no way to turn off the emission of gamma rays. Cobalt-60 is probably more widely accepted in food irradiation processes due to its greater energy than Cesium-137, excellent penetration and it is not water soluble. Cesium-137 is water soluble and

thus the potential for environmental contamination is greater (C.F.R., Title 21, Part 179, 4-1-94).

Other sources of radiation are currently allowed in food irradiation processing in the U.S. They include 1.) X-rays generated from machine sources (linear accelerators) at energies of ≤ 5.0 MeV, and 2.) electron beams generated from machine sources at energies of ≤ 10 MeV (C.F.R., Title 21, Part 179, 4-1-94). In considering the use either of these alternative sources, it should be pointed out that X-rays have a similar penetrating power to gamma rays which both are far greater than electrons. X-rays and electrons both have the advantage of being machine generated and thus have the ability to be turned off and on. Furthermore, electrons can be generated quite efficiently at very high doses and quickly in an accelerator, whereas X-ray production is a process.

Radiation Dose

Perhaps the most important parameter to consider in a food irradiation process is the radiation dose to be delivered. In older literature, the unit used to express radiation dose delivered to a product was the radiation absorbed dose, or rad. One rad is equivalent to 100 ergs of absorbed energy per gram. Newer literature uses the International System of Units (SI) where the unit Gray (Gy) predominates. One Gray is equal to 100 rads and 1 joule of energy absorbed per kilogram of food mass

(Urbain, 1986). Currently, the FDA views doses up to 1.0 kGy as safe and effective for being used in food irradiation processes. This 1.0 kGy dose is one-tenth of the 10 kGy that has been suggested as a safe level by the Codex Alimentarius Committee of the United Nations. It should be noted that 10 kGy is the thermal equivalent of 2.4 calories. The primary exception to this 10 kGy maximum rule occurs with the irradiation of spices, in that they are approved for irradiation up to 30 kGy (C.F.R., Title 21, Part 179, 4-1-94). Gamma irradiation has been proposed as a method of food preservation in that it leads to lethal damage of DNA in food spoilage and pathogenic microorganisms, while not elevating the product temperature (no cooking). Therefore, with little temperature change in the irradiated product, the sensory or quality changes should be insignificant (Kamplemacher, 1983).

The desired effect of irradiation on the food product is certainly a function of dose delivered. Thus, irradiation can lead to a delay in the ripening of a food product, up to complete sterilization of a product. If the exposure is below 1 kGy, food undergoes what is termed radurization. Radurization prevents sprouting in vegetables, delays ripening in fruits and sterilizes insects in grains. If the exposure dose falls within 1-10 kGy, food undergoes what is termed as radicidation. At this level, insects are not only sterilized, but also

killed, and the numbers of bacteria, yeasts and molds in a food product are significantly reduced. If the exposure dose is above 10 kGy, it undergoes what is called radappertization. Radappertization is essentially complete sterilization of a food, as all bacteria and viruses are eliminated (Urbain, 1986).

Dosimetry

A particular concern in oyster irradiation is that the internal structure of the oysters (meats) does not receive the same dose as does the outer shell or outside of the box. The technical staff of FST does provide a determination of the dose delivered to the product, and refer to it as a max/min ratio. This ratio is defined as the maximum absorbed dose that any part of the irradiated product received (D_{max}) divided by the minimum absorbed dose that any part of the irradiated product received (D_{min}), as determined by cerium dosimeters. This ratio is thus a measure of dose uniformity, as it is desirable for all parts of the product to be irradiated at the same dose, thus yielding a ratio of 1.0. However, uniform dose delivery almost never occurs in practice due to factors such as geometry due to the inverse square law (radiation intensity falls off to the square of the distance of the source) and attenuation (bulk density of oyster shells in this case). Thus, researchers are faced with either choosing a maximum or minimum dose to be delivered to the product (Stein, 1995).

The D_{max} is usually referred to as the maximum dose at which no undesirable effects are seen and the D_{min} is referred to as the minimum dose necessary to have the desired effect (i.e., killing of all insects). If the researcher sets a D_{min} and wants to be sure that every part of the product receives that target dose, then the actual dose (D_{max}) that the entire product received will be higher than the target dose, giving a D_{max}/D_{min} of greater than 1.0. This may not be desirable if the actual dose (D_{max}) delivered leads to undesirable effects.

Oyster Irradiation Research

Significant research has been performed on evaluating gamma irradiation as a means for reducing bacterial numbers and increasing the shelf life of oyster and clam meats. However, minimal research has been conducted on shellstock oysters and clams. The pioneering research in this area was conducted by Gardner and Watts (1957) who treated oyster meats at 630, 830 and 3500 rads and observed the development of undesirable "grassy" odors in noncooked, irradiated oyster meats. Furthermore, an "oxidized" odor was detected in the cooked, irradiated oyster meats. Gardner and Watts concluded that ionizing radiation would not be effective in oyster meat preservation, especially since enzyme action continues even at 3500 rads and 5°C storage.

The next significant oyster irradiation research was performed by Novak et al. (1966) who irradiated oyster

meats in cans in air at 2 kGy. The irradiated and nonirradiated oysters were stored on ice for 0, 7, 14, 21 and 23 days. Taste panels determined that the irradiated samples were acceptable throughout the 23 day period, whereas the nonirradiated controls were found to be "spoiled" on day 7 forward (Novak et al., 1966). Next, it was concluded by Slavin et al. (1966) that the optimum irradiation dose for oyster meats was 2 kGy, which when combined with 0.6°C storage, resulted in a shelf life of 21 to 28 days (Slavin et al., 1966). This work was supported by Metlitskii et al. (1968) who showed that oyster meats irradiated at 5 kGy and stored at 2°C, have a shelf life of 60 days.

Liuzzo et al. (1970) investigated what irradiation dose would result in the greatest shelf life of shucked oyster meats, while at the same time resulting in the least alteration in food components. It was determined that 2.5 kGy would extend the shelf life of oyster meats about 7 days. The sensory quality of the irradiated meats was not significantly different from the nonirradiated meats until 7 days of storage on ice. However, doses above 1.0 kGy did alter the retention of B-vitamins in oyster meats, as well as the percent moisture, the percent ash, the glycogen content and the soluble sugar content of oyster meats (Liuzzo et al., 1970).

When considering shellstock oysters, Kilgen et al. (1988) showed that a dose of 1.0 kGy was sufficient to reduce all Vibrio pathogens to undetectable levels and that 1.0 kGy was not lethal to oysters. Furthermore, there were no significant sensory changes in oysters irradiated at this dose (Kilgen et al., 1988). Mallett et al. (1991) irradiated Massachusetts shellstock oysters and found no significant difference in the six day post-irradiation survival times of these oysters at doses up to 2.5 kGy. Mallett et al. (1991) concluded that the median post-irradiation survival time for shellstock oysters was greater than 25 days for exposures of 2.5 kGy and lower. Furthermore, Mallett et al. (1991) utilized a professional taste panel and found that oysters irradiated at 3.0 kGy or less were of fair and acceptable quality.

Mallett et al. (1991) also assessed the effects of ionizing radiation on hepatitis A virus and rotavirus SA11 in oysters and clams. The D_{10} values obtained for hepatitis A virus was 2.0 kGy, and 2.4 kGy for rotavirus SA11. The effects of ionizing radiation on the survival of poliovirus in West Coast shellstock oysters (Crassostrea virginica and Ostrea lurida) was determined years before by DiGirolamo et al. (1972) who found that 400 krads (4 kGy) was effective in reducing the poliovirus by 87% and by 90% in shucked meats. This is equivalent to roughly a 1.0 $\log_{10}$ reduction and thus

higher doses would be necessary to completely eliminate this virus. The only setback is that unacceptable sensory changes begin to develop after such a dose.

Dixon (1992) irradiated Florida shellstock oysters and showed that low doses of gamma radiation (0-3.0 kGy), were not effective in significantly extending the shelf life of oysters beyond that of the nonirradiated controls at 4-6^{o}C. The oysters irradiated at Food Technology Services (formerly Vindicator, Inc.) had D_{50} values of 30, 25, 7 and 6 days and D_{20} values of 17, 9, 4 and 4 days at 0.5, 1.0, 2.0 and 3.0 kGy, respectively. None of these D_{50} values observed for the irradiated oysters exceeded the D_{50} observed for the nonirradiated oysters, which was well over 30 days, or the D_{20} which was 30 days. This is in stark contrast to Kilgen et al. (1988) who found there was significant reduction in Vibrio spp. at 1.0 kGy, and no significant lethality to shellstock oysters.

Clam Irradiation Research

The irradiation of clam meats have also been researched, and results by Nickerson (1963) indicate that clam meats irradiated at 8.0 kGy and stored for 40 days at 6^{o}C had similar sensory scores to the nonirradiated control meats. Nickerson further showed that clam meats exposed to 4.5 kGy also store for 28 days at 6^{o}C. This was corroborated by Slavin et al. (1963) who also found that clam meats exposed up to 4.5 kGy and stored at 6^{o}C

were of equal quality to nonirradiated clam meats. Next, Connors and Steinberg (1964) irradiated clam meats from 2.5 to 5.5 kGy and a taste panel detected no significant difference between the irradiated and nonirradiated meats.

Harewood et al. (1994) evaluated the effects of gamma radiation on the shelf life and bacterial/viral loads in hard-shelled clams (Mercenaria mercenaria). Radiation sensitivity D_{10} values were found to be 1.32 kGy for total coliforms, 1.39 kGy for fecal coliforms, 1.54 kGy for E. coli, 2.71 kGy for C. perfringens and 13.5 kGy for F-coliphage (Harewood et al., 1994). In addition, doses higher than 0.5 kGy were lethal to the shellfish.

Vibrio Vulnificus Radiosensitivity

The extreme radiosensitivity of V. vulnificus was confirmed by Dixon (1992) by calculating the D_{10} value of the 18-24 hour organism in phosphate buffered saline. The D_{10} of the virulent (O) form of V. vulnificus was found to be 0.062 kGy, whereas the D_{10} of the avirulent (T) form of V. vulnificus was found to be 0.037 kGy, when these organisms were plated on both TCBS and CPC agars. These two D_{10} values in V. vulnificus are extremely low, especially when compared to the D_{10} values determined by Licciardello et al. (1989) who found the D_{10} values of Shigella flexneri, E. coli, Salmonella typhimurium Streptococcus faecalis, Staphylococcus aureus, to be

0.35, 0.40, 0.64, 0.85 and 1.00 kGy, respectively, in soft-shell clam homogenate. Even further, the D_{10} values of E. coli, Staphylococcus aureus, Salmonella typhimurium and Streptococcus faecalis were found to be 0.37, 0.42, 0.51 and 0.97 kGy, respectively in oyster mantle fluid (Mallett et al., 1991). Other Vibrio species have been assessed D_{10} values in a variety of media. Matches (1971) and Bandekar et al. (1987) found the D_{10} of V. parahaemolyticus to be 0.10 kGy in shrimp homogenate. Grodner et al. (1990) found the D_{10} of V. parahaemolyticus to be equal to 0.085 kGy in blue crab meat homogenate.

MATERIALS AND METHODS

The first phase of the research included analyzing the effects of low dose gamma radiation on Florida and Texas shellstock oysters in terms of microbiological consequences, shelf life and identification of surviving organisms, shell to meat ratio and dosimetry inside the shell.

Source of Oysters

There were two primary sources of shellstock oysters used in this research. The first was Leavins Seafood, Inc. in Apalachicola, FL and the other was Northwest Seafood, Inc. in Gainesville, FL. Both provided oysters thoughout the course of this study. However, most importantly, Leavins provided the Florida and Texas oysters for the large scale irradiation of oysters at Food Technology Services, while Northwest Seafood provided the use of a refrigerated truck for the transport of those oysters for irradiation. The actual harvest area of the oysters was Catpoint in Apalachicola Bay, FL and Galveston Bay in Galveston, TX.

Irradiation Sources

One source of gamma irradiation was a gamma ray emitting unit using the radionuclide ^{137}Cs. The unit is located in the Entomology Research Laboratory on the

campus of the University of Florida, Gainesville, FL. There were certain limitations with this unit in that samples to be irradiated were limited to 6 in^3. Thus, much of the pure culture and VBNC cell work that was contained within a test tube was performed in this unit.

The other source of irradiation was the 3.0 million curie ^{60}Co unit located at Food Technology Services, Inc. in Mulberry, FL (formerly Vindicator, Inc.). This facility is located approximately 135 miles due south of Gainesville, FL and is equipped to handle commercial amounts of products (i.e., semi-tractor trailer loads). Thus, this was the facility used for the large scale irradiation of shellstock oysters.

Large Scale Irradiation

A large, commercial scale irradiation at Food Technology Services, Inc. (FTS) was performed on 33 bushels of oysters from Florida and Texas. The breakdown of how the oysters for the shelf life and microbiology experiments were divided is shown in Table 2.

Transportation of Shellstock to FTS

Thirty three bushels (≈ 2000 lbs./909 kg) of shellstock oysters were transported by refrigerated truck to Food Technology Services, Inc. in Mulberry, FL (≈ 135 miles). The temperature range experienced by these shellstock was between 4°C to 7°C. Upon arrival, the oysters were unloaded into a holding cooler (4-6°C) that

was located on the premises. Food Technology Services, Inc. set no limitations on the number of oysters that could be irradiated. Thirty-three bushels (≈ 150 - 200 shellstock oysters in each bushel) of oysters were separated as shown in Table 2 and irradiated at two different doses. Some samples were set aside as nonirradiated controls.

Table 2. The breakdown of the 33 bushels of FL and TX oysters by source and experimental treatment groups.

	FLORIDA		TEXAS	
	Shelflife	Microbiology	Shelflife	Microbiology
Control	5 (850)	2 (≈ 300)	3 (500)	1 (≈ 150)
1 kGy	5 (873)	2 (≈ 300)	3 (513)	1 (≈ 150)
3 kGy	5 (865)	2 (≈ 300)	3 (507)	1 (≈ 150)

Number indicates bushels of oysters, and number in parentheses indicates total number of oysters.

The two different maximum/minimum doses that were delivered to the shellstock oysters were 0.99 kGy to 0.50 kGy (T_1) or 2:1, and 2.99 kGy to 1.99 kGy (T_3) or 1.5:1. These doses were determined by the technical staff of FTS using cerium dosimeters. After irradiation, all 33 bushels were transported back to the University of Florida by refrigerated truck and stored at 4-6°C in wax-covered, cardboard boxes. These boxes are identical to those used by the shellfish industry for the transport and dry storage of shellstock oysters and clams. Separate batches of shellstock oysters were irradiated

for the shelf life analysis and the microbiological analyses to avoid any selectivity problems.

Microbiological Analyses

Oysters for the microbiological study were sampled according to the accepted practices of the Interstate Shellfish Sanitation Conference (ISSC). According to the National Shellfish Sanitation Program (NSSP), waters receive approved status for harvesting shellfish as long as the fecal coliform standard MPN does not exceed 14 CFU per 100 mL seawater and no more than 10% of the samples tested can exceed 43 CFU per 100 mL in a 5-tube MPN. Furthermore, fresh and frozen meats can not have a fecal coliform count of greater than 2300 per 100 grams or a 35°C plate count of greater than 50,000,000 per 100 grams meat (NSSP, 1990a, b). The State of Florida, Department of Environmental Protection's Shellfish Sanitation Division also follows such practices.

Microbial analysis was conducted immediately after irradiation and subsequently during dry storage. Shellstock oysters were scrubbed with a brush and rinsed under running tap water and subsequently shucked under aseptic conditions. Approximately 10-12 clean, single oysters were shucked and placed into a sterile Waring™ blender. This meat was homogenized for 1½ minutes on high speed and 11 grams of this homogenate were serially diluted into 99 mL of phosphate buffer water out to 10^{-8}.

Most probable number (MPN) tubes, in triplicate, were inoculated first. These MPN tubes consisted of 9.0 mL of alkaline peptone water (APW) at 2.5% NaCl and pH = 8.4, and they were inoculated with 1.0 mL from each dilution. APW is an enrichment medium in which Vibrios proliferate quite and outgrow other contaminating microorganisms due to the high salt concentration and high pH (Sloan et al., 1992). Tubes were diluted out to the 10^{-8}. Once inoculated, the MPN tubes were then incubated at 37°C for 8-12 hours (Sloan et al., 1992). Tubes were checked for growth/turbidity, which is indicative of a positive result and recorded. These positive or negative results yield a three digit code number that was cross-referenced in a MPN table, and thus provided a statistical count of bacteria (Standard Methods, 1992). All positive tubes were then streaked aseptically onto thiosulfate-citrate-bile salts-sucrose agar (TCBS) and colistin-polymixin-cellobiose agar (CPC) (BAM, 1994). TCBS agar is selective for "Vibrio-like" organisms (Lotz et al., 1983) and CPC agar is selective for V. vulnificus (Massad and Oliver, 1987; Sloan et al., 1992; Sun and Oliver, 1995; Kaysner and Tamplin, 1988; Kaysner et al., 1989). After streaking, TCBS plates were incubated for 24 hours at 37°C (Lotz et al., 1983), and observed for growth. CPC plates were incubated for 24 hours at 42°C, and observed for growth. CPC plates are incubated at this elevated temperature of 42°C because

most other organisms are inhibited at this high temperature, especially V. parahaemolyticus (Massad and Oliver, 1987). CPC has also been shown to be more selective and effective than the corresponding Vibrio vulnificus enrichment (VVE) medium (Miceli et al., 1993; Sun and Oliver, 1995). The number of plates exhibiting growth (positive) were compared to the number of positive tubes from the MPN test, and thus positive plate counts from TCBS and CPC can be constructed into an MPN, similar to the MPN tubes.

One mL samples from the serial dilution bottles were pour plated on total plate count agar (PCA) and on plate count agar with 2.5% NaCl (PCAS) in duplicate. These two plating media gave an assessment of the total aerobic plate count and a determination of the number of the strict halophilic organisms in the oysters. This plating regime goes beyond the State of Florida testing methodology, which does call for PCAS, thus potentially missing a whole population of organisms (strict halophiles). It would seem logical to plate on a salt containing media, since bacteria are living and growing in seawater, which does contain salt. Likewise, 1 mL samples from the serial dilutions were inoculated into lactose broth (LB) tubes (5 tubes per dilution) and incubated at 37°C. Similar to the APW MPN above, the positive LB tubes (gas and growth) can be used as an MPN to give a count of the total number of coliforms. One mL

samples from all positive LB tubes were transferred to EC-MUG tubes and incubated at 44.5°C. Positive tubes are distinguished by growth and gas production, as well as flouresce under a UV lamp. Positive tubes were counted as an MPN to give a total count of E. coli.

Shelf Life

During each day of storage, all of the oysters were individually picked up by hand and examined for death. If the oyster was found to be gaping open, it was squeezed by the thumb and index finger. If the shellstock oyster remained closed after squeezing, it was considered alive. If it popped back open upon release by the thumb and index finger, it was considered dead. All dead oysters were removed from the boxes and the survivors were returned to the cold dry storage conditions. Oysters were examined and counted nearly every day for 14 days. A two week time period was selected since this is the maximum allowable time between harvest and sale of oysters. The values obtained in the shelf life study were statistically analyzed by a Chi-Square Test (X^2) performed at the 99% confidence interval. This type of count or enumerative data has characteristics that define a multinomial experiment, and thus the X^2 test is the most appropriate test statistic for determining when the death rates become significantly different.

Shell to Meat Ratio

The actual shell density of oysters from different geographic regions may vary. Thus, it is necessary to determine the shell weight and meat weight of oysters from FL, TX and LA. Approximately 100 oysters each from the FL and TX oysters of the irradiation study were washed and scrubbed under tap water, weighed, shucked completely and then the remaining shell weighed again. This method allowed for a shell weight to meat weight ratio to be calculated. Louisiana oysters were prepared similarly on a separate occasion. This was done in concert with the internal shell dosimetry.

Shell Dosimetry

It was determined that for this research a maximum value of 1.0 kGy and 3.0 kGy should be set since these were the doses that were pending approval before FDA. Thus, we had no control of the D_{min}; however, it was stressed to the technical staff that a D_{max}/D_{min} of as close to 1.0 be reached, since a large variation in dose is not acceptable for oysters. The goal of this phase of the study was to compare the D_{max}/D_{min} ratios provided by FST with some internal dosimeters of our own. Oyster shells were shucked of their meats and put back together, held by a rubberband. A dosimeter previously rapped in waterproof plastic rap was placed inside of FL and TX oysters and placed back into the bushel boxes for irradiation at 1.0 and 3.0 kGy. They were placed in the

center of the box, surrounded by other oysters. After irradiation, dosimeters were read in a spectrophotometer and the dose delivered calculated by the appropriate National Standards of Radiation Dosimetry.

Surviving Organisms

After incubation of these agar plates and tubes, any isolated, surviving organisms were identified using the API 20e™ Biochemical Identification System of Enterobacteriaceae. The methodology consists of inoculating a series of different chemical substrates in cupules with a saline suspension of bacteria and then measuring the biochemical use by color changes and addition of chemical reagents. The biochemical profile generated can be used to identify different Gram-negative bacteria and Enterobacteriaceae.

Preparation of Artificial Seawater Microcosms

Artificial seawater (ASW) was prepared using the commercially available artificial seasalt Instant Ocean™. This seasalt was rehydrated in distilled water to a specific gravity of 1.022 or 30 parts per thousand (ppt). This solution is loaded with particulate matter, thus it twice vacuum filtered, first through Whatman 0.45 μm filter paper, followed by another filtration through a 0.22 μm Millipore filter. Seven hundred fifty mL of this filtered seawater were then placed in 1 L flasks that were previously prewashed with 6N HCl to remove all

organic material. They were autoclaved at 121°C for 15 minutes and kept refrigerated until use. This double filtered and sterilized seawater was used as the seawater in the microcosms, diluent for cell washing and in all serial dilutions.

Preparation and Maintenance of Bacterial Cultures

Two different strains of V. vulnificus were used in this study. The first strain is the C7184 strain of V. vulnificus (both O and T) which was supplied by Dr. James Oliver of UNC-Charlotte. The other strain was CVD 713 which has been genetically altered by Dr. Glenn Morris of the University of Maryland and is generically referred to as the "Blue-bug" (B). This strain is a true O morphotype, however this strain has a single insertion transposon for alkaline phosphatase and kanamycin resistance (Tn*PhoA*). The E. coli gene coding for alkaline phosphatase, minus the promoter and signal sequences, was fused in frame to a gene encoding a secreted protein, which yields an active alkaline phosphatase enzyme (Manoil and Beckwith, 1985). The Tn*PhoA* was introduced into MO6-24 by conjugation of the vector pRT291 in E. coli SM-10 with selection on media containing polymyxin B and kanamycin (Wright et al., 1990). The cells can then be selectively grown on TN agar, which yield blue colonies. The blueness of the colonies is due to the presence of 5-bromo-4-chloro-3-indoyl phosphate in the media. This is the chromagenic

substrate for the enzyme alkaline phosphatase. The media is selective for only those colonies with kanamycin resistance. All of the cells used in the experiment were maintained on alkaline peptone agar at pH = 8.4 (APA) and heart infusion agar at 2.5% NaCl (HIS) at room temperature.

Inducing VBNC Vibrio vulnificus

The viable but non-culturable (VBNC) form of V. vulnificus was induced in the O's, T's and B's so that the influence of ionizing gamma radiation (^{137}Cs) could be determined. One colony forming unit (CFU) from the cells maintained on either APA or HIS was used to inoculate heart infusion broth at 2.5% NaCl (HIBS). The cells were cultured in 200 mL of HIBS overnight at room temperature. After incubation, this broth was centrifuged at 10,000 X g for 20 minutes. The supernatant was discarded and the pellet was washed and resuspended in artificial seawater (ASW). This resuspension was centrifuged and washed 2 more times under the same parameters. Once a 2X washed pellet was obtained, the supernatant was discarded, and the resulting pellet was resuspended in approximately 50 mL of ASW. This final resuspension was added directly into the pre-prepared, room temperature 750 mL ASW microcosms. These ASW microcosms were then sampled to get an original bacterial count and then placed in the refrigerator at 4°C. The microcosms were sampled for culturability by performing serial dilutions (1 mL:9 mL)

in ASW and spread plating 0.1 mL aliquots on nonselective media such as HIS or APA, as well as selective media such as TCBS for "Vibrio-like" organisms and CPC for V. vulnificus. Another medium, TN agar, was also used when the "blue-bug" (B) or CVD 713 strain was used. Culturable counts were made frequently over the 4 week period, while the VBNC state was being induced with special care taken not to contaminate the microcosms. When culturability decreased to less than 10 CFU/mL, 1 mL aliquots were plated. Once these media no longer showed growth, the next test was to place a 10 mL sample of the microcosm into 3 tubes each of 10 mL of double strength alkaline peptone water (DS-APW) and incubate at 37°C for 24 hours. This was done daily until the DS-APW was no longer positive after incubation. Once the cells would no longer grow on liquid or solid media, the cells were assumed to be nonculturable (FDA, 1989).

Total bacterial counts were also made on the microcosms using the acridine orange direct count (AODC) using an epifluorescence microscope (Hobbie et al., 1977). Two mL of the microcosms were removed, placed in a sterile test tubes and fixed with 106 µL of 2% formalin (final concentration). Following fixation, the cells were diluted 100- to 1000-fold, stained with 200 µL of acridine orange in 66 mM phosphate buffer. The stained samples were then filtered through a prestained Irgan

black 0.2 μm Nucleopore polycarbonate filter (Millipore). This filter was placed on a glass slide, and topped with a drop of microscope oil. Very carefully, a glass cover slip was placed on top of the filter, and another drop of oil placed on the cover slip. The slide and filter were viewed under a microscope, and all fluorescing bacteria are counted using an oil immersion, 100x lens, Nikon-Microcomp microscope with a Super High Pressure Mercury Lamp (Model HB-10101AF).

The AODC method is limited in that it will pick up any cells that are present in the sample, dead or alive. Thus, some means for detecting only actively metabolizing cells must be employed. This is accomplished with the DVC method using nalidixic acid and yeast extract (Kogure et al., 1979). In this procedure, a 2.0 mL sample is enriched in 20 μL of 0.025% (w/v) yeast extract, to which 20 μL of 0.002% (w/v) nalidixic acid is also added. This mixture is incubated in the dark at room temperature for 2-3 hours for fresh cultures (overnight for cells closer to the VBNC state). This is an alteration of Kogure's methods, who suggests a 6 hour incubation regardless of cell age. However, Birbari and Rodrick (1990) showed that about 12 hours was a better incubation time for cells closer to the VBNC state and that 2-3 hours was better for fresh cultures. The reason for the decreased incubation time stems from 6 hours being too long for

cells to elongate, and breakage may sometimes occur, which will lead to higher cell counts. Twelve hours was also suggested by Birbari and Rodrick (1990) as the proper time for incubating VBNC cells to allow more time for cells to respond to the yeast extract. After incubation, the cells are fixed with 106 μL of formalin, followed by subsequent staining for two minutes wth acridine orange. After staining, filtering and slide preparation are conducted as described above for AODC. Those cells that respond to yeast extract exhibit cell elongation; however, cell division does not occur due to the presence of nalidixic acid. It is the elongated cells that are considered still metabolically active and are counted. The spherical cells that remained after incubation (thus did not elongate) are considered dead cells and not counted.

For each AODC and DVC experiment, a minimum of ten microscopic fields were counted for each sample. A micrometer was used to measure the diameter of each microscopic field (d=0.14 mm and r=0.07 mm) and thus the area calculated.

$$F = \frac{\text{area of filter}}{\text{area of microscopic field} \times \text{volume of sample filtered}}$$

$$F = \frac{12.5 \text{ mm} \times 12.5 \text{ mm} \times 3.14}{0.07 \text{ mm} \times 0.07 \text{ mm} \times 3.14 \times 2 \text{ mL}}$$

$$F = 15,944$$

Using the diameter of the filter as 25 mm (r=12.5 mm) and the volume of cells as 2.0 mL, the number cells found in the original sample can be calculated by the conversion factor F.

Resuscitation of VBNC Vibrio Vulnificus

After inducing the VBNC state in V. vulnificus, it is always necessary to assess whether or not culturability returns after "warming up." Thus, 2-5 mL samples from the microcosms (after negative in DS-APW) were incubated at room temperature for 24 and 48 hours, and were subsequently serially diluted (1mL:9mL) in ASW. Culturability was determined by plating 0.1 mL of tempered sample on HIS, APA, TN, CPC and TCBS agars. Normally these agars would be incubated at 37°C, but to minimize the stress on these VBNC cells, they were incubated at room temperature instead. Furthermore, direct viable counts (DVC) and total cell counts (AODC) were also performed.

Resuscitation experiments were also conducted with consideration taken for the effects of dilution. The irradiated samples (2 mL) were allowed to resuscitate and furthermore these cells were diluted 10-, 100- and 1000-fold and checked for resuscitation. If resuscitation occurs, then these samples would have 10-, 100- and 1000-fold less bacteria than the undiluted samples. Similarly, resuscitation was carried out in the presence of nalidixic acid. The presence of this compound

prevents DNA synthesis and hence cell division. This prevents any few remaining culturable cells from growing and dividing and thus indicating resuscitation when it really did not occur. With added nalidixic acid, only cells that are truly resuscitating would then grow on solid media. One final experiment consisted of taking full microcosms (750 mL) of VBNC cells, centrifuging at 10,000 x g for 20 minutes, leaving a pellet of VBNC cells. These cells were washed 2X in fresh ASW and resuspended in fresh ASW. The cells were incubated at room temperature for 24 and 48 hours and observed for resuscitation.

Cell Culture Irradiation Protocol

All cells regardless of form (log phase O, T and B, stationary phase O, T and B or VBNC O, T and B) were irradiated in a similar manner. Two mL samples were placed into sterile test tubes, and kept on ice. These tubes were placed around the inner circumference of an ice-filled 500 mL beaker. The samples were irradiated at specific doses, and the numbers of survivors determined. For the normal cells (log and stationary phase O, T and B) after irradiation, serial dilutions were made for each of the doses, and plating was done on a variety of media including APA, TCBS, CPC and TN (for "blue-bug" only). For the VBNC V. vulnificus (O, T and B), cell counts were determined immediately after irradiation by AODC and DVC. Furthermore, irradiated VBNC cells were incubated at room

temperature for 24 and 48 hours, and then checked for resuscitation on APA, TCBS, CPC and TN agars, as well as DVC and AODC. This cell irradiation differs from previous research in this area in that ASW is the medium and not phosphate buffered saline (PBS), a distinction between log and stationary phase culturable cells is made, and VBNC cells are being irradiated for the first time.

After counts were made, survival curves were constructed by a plot of the surviving percentage of V. vulnificus versus irradiation dose. These curves were fitted by linear regression and V. vulnificus radiosensitivity was expressed as a D_{10} value. It is the D_{10} value that is the most widely accepted term for assessing radiosensitivity. A D_{10} value is defined as the dose required to inactivate a population of bacteria to 10% of its initial value. It is obtained by taking the negative inverse of the slope of the straight line portion of the survival curve (Urbain, 1986).

RESULTS AND DISCUSSION

Large Scale (Commercial) Irradiation

The primary goal of this research focused on determining the effects that gamma radiation had on FL, LA and TX shellstock oysters in terms of microbial reduction and enhanced shelf life. Only FL and TX oysters were included in the study as Louisiana oysters were not provided as promised on the actual day of irradiation. Figures 1-17 and Tables 3-5 demonstrate the data obtained from this experiment.

Microbiological Analysis

Figures 1-14 show microbiological data associated with nonirradiated oysters and oysters irradiated at 1.0 and 3.0 kGy over a two week dry storage time, with plating on a variety of different media. Figure 1 shows the bacterial counts obtained on PCA from Apalachicola Bay, FL shellstock oysters. Notice that the levels are below the established guidelines of total plate count set by the National Shellfish Sanitation Program (NSSP, 1990a, b). On day 0, immediately following irradiation, the 1.0 kGy and 3.0 kGy counts are reduced by about 1 log and 2.5 logs respectively. These immediate reductions in bacterial counts are indicative of the ability of radiation to cause microbial lethality. However, on day 2

of dry cold storage, the bacterial counts in the irradiated oysters were beginning to rise, with the 1.0 kGy (A_1) oysters showing about a 1 log increase and the 3.0 kGy (A_3) showing about a 1.5 log increase. At this point, the 1.0 kGy exposure has almost as many bacteria, as do the nonirradiated controls which have not increased significantly since day 0.

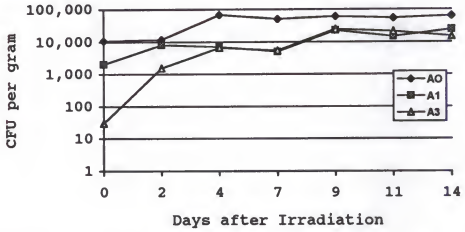

Figure 1. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plated on plate count agar (PCA) over a 2 week period.

Day 4 shows about a 1.0 log increase in counts obtained from the nonirradiated controls, and these control counts are about 1 log higher than than the A_1 and A_3 groups. The A_1 group is roughly the same as day 2, but the A_3 group has increased another 1.0 log up to that count observed in the A_1. On day 7, the counts remain within the same log cycle as day 4 and day 9 shows about a 0.5 log increase in the A_1 and A_3 groups. These irradiated oysters stay within about 0.5 logs of the

control throughout the remaining five days of dry cold storage.

Figure 2 shows the bacterial counts obtained on standard plate count agar plus 2.5% NaCl (PCAS) on Apalachicola Bay, FL shellstock oysters over two weeks of dry cold storage. This allowed for a differentiation of halophiles and nonhalophiles in the shellstock oysters.

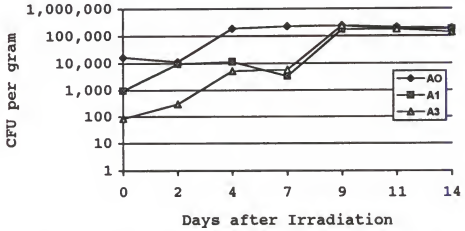

Figure 2. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plated on plate count agar with 2.5% NaCl (PCAS) over a 2 week period.

On day 0, immediately following irradiation, the 1.0 kGy and 3.0 kGy counts are reduced by about 1.0 log and 2.0 logs, respectively. Notice that the counts obtained on PCAS in the A_3 group are about 0.5 logs higher than that was obtained on PCA, indicating a substantial population of halophiles exists in the shellstock oysters. The increase in bacterial counts obtained on PCAS were similar to those detected on PCA, however the counts on PCAS exceeded 1.0×10^5 CFU per gram which

never occurred on the PCA plates. Day 2 exhibited similar counts to that observed on PCA; however, this deviated on day 4. On day 4, the control values exceeded 1.0×10^5 CFU per gram, but the A_1 and A_3 groups remained similar to what was observed on PCA. Day 7 data was similar to day 4, but on day 9 all three groups exceeded 1.0×10^5 CFU per gram which is 0.5 to 1.0 logs higher than the PCA values. This trend continued on days 11 and 14. This would tend to indicate that the halophilic organisms are able to grow and divide post-irradiation, possibly due to a lack of competition from other nonhalophiles that were killed by irradiation.

Figure 3 shows the bacterial counts obtained in an alkaline peptone water most probable number (APW-MPN) on Apalachicola Bay, FL shellstock oysters over a two week period. It should be pointed out that APW-MPN is a mild resuscitation media that allows for slow recovery of cells. Cells are allowed to resuscitate in a mild broth for 8-12 hours and thus may be expected to have a higher count than that observed on traditional solid plate count agar. The tubes that exhibit positive growth are considered presumptive Vibrios.

On day 0, immediately following irradiation, both the 1.0 kGy and 3.0 kGy counts are reduced by about 0.5 log and 1.0 log respectively. The data show an overall increase in numbers across the two week period. The control values on APW-MPN remained about 1.0 log higher

than the A_1 and A_3 exposures throughout the 14 days of storage. The data shown in Figure 3 agree quite well with the data on PCAS, as the counts in the control exceed 1.0×10^5 and actually get near 1.0×10^6 CFU per gram. The A_3 oysters however do not exceed 1.0×10^5 CFU per gram as shown in Figure 2, but the A_1 group does on day 14. Thus, the counts in the irradiated oysters never reached those counts observed in the controls, as was seen on PCAS.

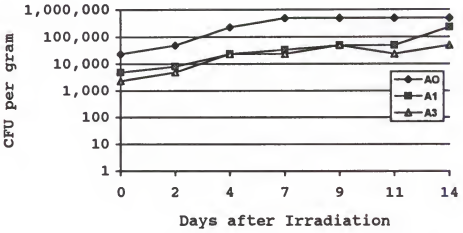

Figure 3. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plating in a 3-tube alkaline peptone water most probable number (APW-MPN) over a 2 week period.

Data in Figure 4 are an extension of the data in Figure 3. As mentioned in the methods section, all positive APW-MPN tubes, presumptive Vibrios, were streaked onto TCBS and CPC agars to yield "Vibrio-like" and V. vulnificus counts, respectively. Thus, the values presented in Figures 4 and 5 should be similar to those in Figure 3. In Figure 4, the numbers of "Vibrio-like"

organisms were determined in the shellstock oysters post-irradiation. The counts on TCBS for all three groups were identical to the APW-MPN counts for the first two days after irradiation, indicating that the presumptive Vibrios were in fact "Vibrio-like." The TCBS counts from the nonirradiated oysters levelled off and stayed at the same for days 2 through 9, with a corresponding increase in counts in the irradiated oysters. This increase reached the level of the nonirradiated controls on day 9 and stayed within a 0.5 log cycle of the controls on days 11 and 14.

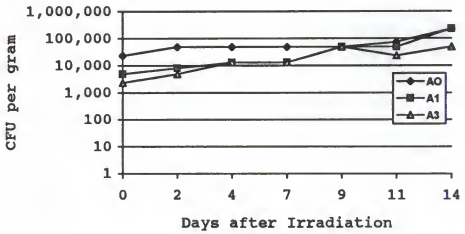

Figure 4. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plating on TCBS over a 2 week period.

Figure 5 shows the numbers of V. vulnificus counted in nonirradiated and irradiated oysters over 2 weeks of dry storage. Again, the counts observed on the first 2 days were identical to those obtained on APW-MPN and TCBS. Similarly, the control value remained relatively

unchanged during days 2-9, with approximately a 0.5 log increase observed across days 9-14. On day 14, the V. vulnificus population was representing 100% of the positive APW-MPN counts in the A_1 group, while the V. vulnificus count was about 1 log lower than that in the A_3 group. When comparing Figures 3-5 to Figures 1-2, it becomes apparent that "Vibrio-like" species and V. vulnificus represent the predominate species in the total plate counts and especially in the PCAS. Furthermore, there is evidence of the growback of "Vibrio-like" and V. vulnificus at 4°C over time.

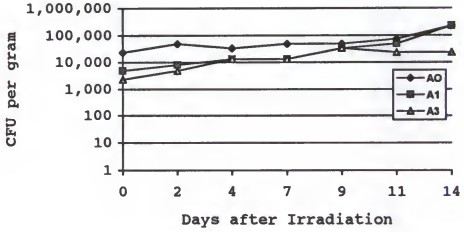

Figure 5. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plating on CPC over a 2 week period.

Figures 6 and 7 show results from lactose broth and EC-MUG. These media are used for the detection of total presumptive coliforms and fecal coliforms (E. coli) in shellfish meats. These two tests, along with the total plate count, are the only tests used in the sampling of

shellfish meats and seawater in the State of Florida. Depending on the counts determined in these tests, shellfish harvesting areas are classified as either approved, restricted or conditionally approved. It should also be remembered that the presence of these bacteria are not indicators of the presence of "Vibrio-like" species (Tamplin et al., 1982; Rodrick et al., 1984). Figure 6 shows the total number of presumptive coliforms present in Apalachicola Bay, FL shellstock oysters after irradiation during 2 weeks of dry storage.

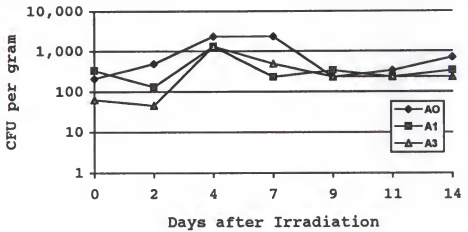

Figure 6. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plating in a 5-tube lactose broth most probable number (LB-MPN) over a 2 week period.

Immediately after irradiation, there were more total coliforms in the A_1 exposure than in the nonirradiated controls, but this trend changed days 2-7. The overall numbers of presumptive coliforms in the oysters is approximately 300 CFU per gram, representing a small fraction of the overall total plate count. As mentioned,

the total plate count is mostly represented by the "Vibrio-like" species, namely V. vulnificus. The A_3 group is reduced to below 100 CFU per gram and both of the groups are below the nonirradiated controls until day 4. On day 4, all three experimental groups have increased in total coliform counts to above 10^3 CFU per gram. This trend continues on day 7 with the control group, but the A_1 and A_3 groups fall below 500 CFU per gram. On days 9, 11 and 14, all three groups stay below 1000 CFU per gram, with the control value the highest on each day.

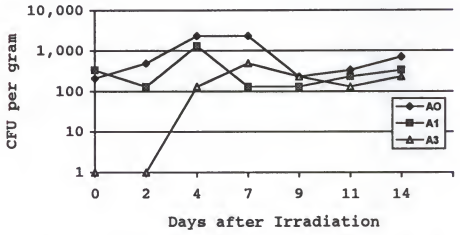

Figure 7. The number of colony forming units (CFU) per gram of oyster meat from Apalachicola Bay, FL shellstock oysters irradiated at 0.0 kGy (A0), 1.0 kGy (A1), and 3.0 kGy (A3), and plating in a 5-tube EC-MUG most probable number (MPN) over a 2 week period.

The data in Figure 7 give an indication that E. coli, a fecal coliform, represents the highest fraction of total presumptive coliforms in these oysters. On day 0, the A_1 count was higher than the control value, but there were no E. coli present in the A_3 group.

Subsequently on day 2, there were no E. coli present in the A_3 oysters. But on day 4, E. coli begins to represent a fraction of the total coliform count and this continues to increase throughout the 14 day analysis. However, it should be pointed out that overall, E. coli represents a very small fraction of the total aerobic plate count. However again, the growback and multiplication of these organisms exists post-irradiation and this can be a potentially dangerous situation.

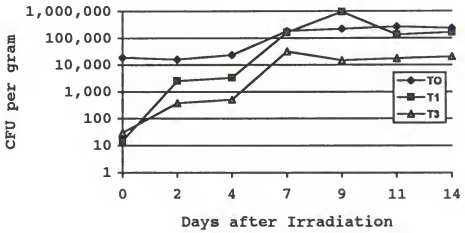

Figure 8. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plated on plate count agar (PCA) over a 2 week period.

This same type of microbiological analysis was done for 14 days on the Galveston Bay, TX shellstock oysters using the same media and storage conditions. Figure 8 shows the total plate counts of irradiated TX shellstock oysters over a 14 day storage time. Immmediately following irradiation, the T_1 and T_3 exposures are reduced about 3.0 log cycles to below 50 CFU per gram.

The plate counts obtained for the T_1 and T_3 exposures begin to increase over days 2 and 4, but still stay 1-2 logs below the nonirradiated control.

On day 7, the T_1 exposure actually reaches that of the nonirradiated control at above 1.0×10^5 CFU per gram, whereas the T_3 exposure stays 1.0 log behind. The T_1 exposure even surpasses the control group on day 9, at nearly 1×10^6 per gram, but falls back below the control on days 11 and 14. The T_3 exposure stays 0.5 to 1.0 log below the control and T_1 expsoures on days 9, 11 and 14.

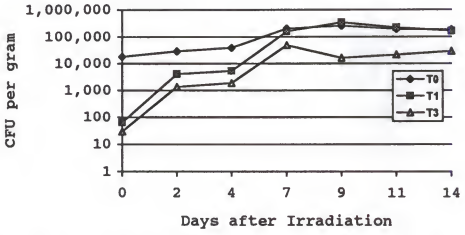

Figure 9. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plated on plate count agar with 2.5% NaCl (PCAS) over a 2 week period.

Figure 9 assesses the halophiles present in the TX shellstock oysters. The numbers immediately after irradiation were quite similar to those obtained on PCA, with the nonirradiated control oysters being >2.0 logs greater than the irradiated oysters. On days 2 and 4, the controls are only 1.0 log higher than their

irradiated counterparts, but this changes on day 7. On day 7 the T_1 exposure increases to nearly that of the control, with the T_3 exposure 0.5 logs behind. On day 9, the T_1 exposure exceeds the control and the T_3 exposure is a full log cycle below. This day 9 trend continues on days 11 and 14.

The data in Figure 10 show the resuscitation of presumptive Vibrio organisms by an APW-MPN. There is approximately a one log reduction observed at the T_1 and T_3 exposures. The counts on this media are higher than those observed on the total plate count. Again, this media is a mild resuscitation media that allows for slow recovery of cells and may be expected to have a higher count.

This trend continues on day 2, but the T_1 exposure is only about 0.5 logs below the control. On day 4, the nonirradiated control count jumps to above 2.0×10^5 CFU per gram with the T_1 and T_3 exposures a full log cycle behind. From day 4-14, the T_3 oysters stay at a constant 2.3×10^4 CFU per gram. The control and the T_1 oysters continue to increase on day 7 and are within a 0.5 log cycle of each other. On day 9, the control and T_1 exposure are reduced from day 7, but are still very close to one another. This trend continues over days 11 and 14.

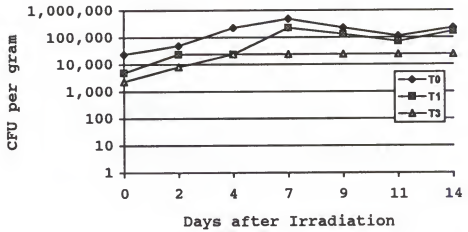

Figure 10. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plating in a 3-tube alkaline peptone water most probable number (APW-MPN) over a 2 week period.

The positive APW-MPN tubes were streaked onto TCBS and CPC agars, and the data obtained is presented in Figures 11 and 12, respectively. All of the positive tubes immediately after irradiation and on day 2, were also positive on TCBS. This was not the case on day 4, when the TCBS value was about 1.0 log less than the corresponding APW-MPN value. Still, the growback of the irradiated oysters does not exceed that of the nonirradiated controls. On day 7 however, the T_1 exposure reaches the same "Vibrio-like" count as the nonirradiated control and stays very close to it on days 9, 11 and 14. Just as in the APW-MPN, the "Vibrio-like" counts reach 2.3×10^4 per gram on day 7 in the T_3 group, and stay at that level for days 9, 11 and 14.

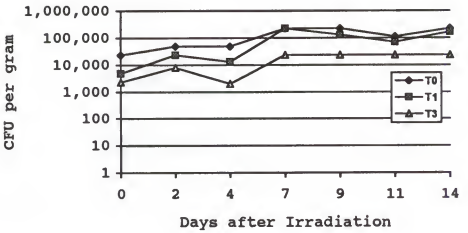

Figure 11. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plating on TCBS over a 2 week period.

Figure 12 gives the numbers of Vibrio vulnificus present in the shellstock oysters over time. Again, all positive tubes in the APW-MPN were also positive on CPC on days 0 and 2. On day 4, the CPC value is extremely low at 2.0×10^3 CFU per gram, and represents a very small fraction of the overall presumptive Vibrios and "Vibrio-like" organisms. The numbers of V. vulnificus in the T_1 and T_3 exposures stay below the numbers in the control throughout the 14 days, but the T_1 exposure gets very close in number on days 11 and 14. Similar to the APW-MPN and TCBS results, the T_3 exposures reaches 2.3×10^4 V. vulnificus cells per gram of oyster meat at day 7 and remains there at days 9, 11 and 14, a full log behind the control and 0.5 logs behind the T_1 exposures.

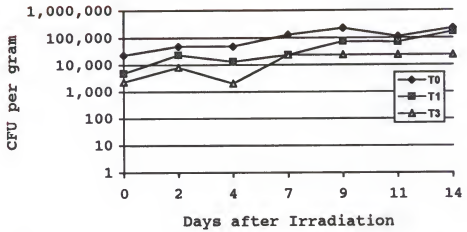

Figure 12. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plating on CPC over a 2 week period.

Figures 13 and 14 give a count of the total presumptive coliforms and fecal coliforms (E. coli) present in the TX oysters over time. The total coliform counts observed were quite low, below 300 CFU per gram, on day 0, with none detected in the T_3 dose. On day 2 however, all three samples had a count of 490 CFU per gram, twice as large as the day before. On day 4, the control count has risen to 1.3×10^3 CFU per gram, with T_1 and T_3 exposures at 0.5 to 1.0 log cycle lower. This control number rises to 2.3×10^3 CFU per gram and the T_1 and T_3 exposures are a full log cycle lower. Days 9 and 11 show all three groups below 330 CFU per gram and this remains on day 14 for the T_1 and T_3 exposures. However, the control value rises to 1.4×10^3 CFU per gram on day 14, a full log cycle higher.

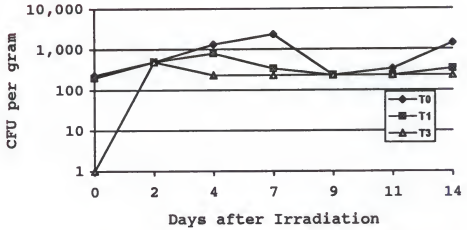

Figure 13. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plating in a 5-tube Lactose Broth most probable number (LB-MPN) over a 2 week period.

In Figure 14, there were no E. coli detected in the T_3 sample, whereas there were around 200 CFU per gram in both the control and T_1 sample on day 0. Day 2 was quite interesting in that all three samples reached a level of nearly 500 CFU per gram, indicating that E. coli was representing all of the total presumptive coliform content. On day 4 the T_3 group was 1 log lower than the control and this continued on day 7. From day 4 on, the T_3 group remained between 130 and 230 CFU per gram. The only abberration arose on day 9 when all three samples yielded identical results at 230 CFU per gram. All three samples were within the same log cycle on day 11 and the control jumped a log cycle higher than T_1 and T_3 exposures on day 14.

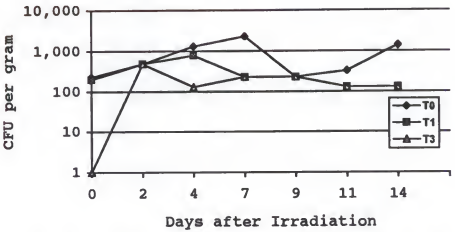

Figure 14. The number of colony forming units (CFU) per gram of oyster meat from Galveston Bay, TX shellstock oysters irradiated at 0.0 kGy (T0), 1.0 kGy (T1), and 3.0 kGy (T3), and plating in a 5-tube EC-MUG most probable number (MPN) over a 2 week period.

Shelflife Analysis

The FL and TX shellstock oysters were also evaluated in terms of shelf life. Figures 15-17 are graphs that show the percentage of surviving oysters plotted against the number of days during cold dry storage at 4-6°C. In Figure 15, the percentage of surviving Apalachicola Bay, FL irradiated and nonirradiated oysters is plotted and compared. The three populations behave quite similarly immediately following and 1 day after irradiation. However, on day 2 the 3.0 kGy group becomes significantly different from the control and 1.0 kGy groups, as detected by a Chi-Square test performed at the 99% confidence level. Similarly, the 1.0 kGy group becomes significantly different from the control group on day 4, as detected by a Chi-Square test at the 99% confidence level. The irradiated oysters continue dying at a rapid

pace. By day 14, no 3.0 kGy oysters were left. It took 22 days for all of the 1.0 kGy oysters to die, and it this point only 36% of the nonirradiated oysters had died.

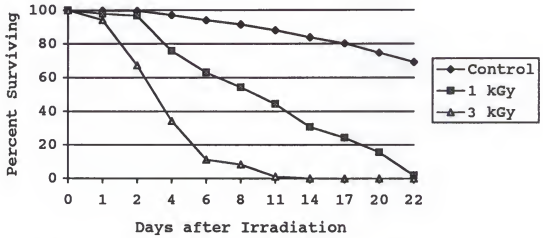

Figure 15. The percentage of Apalachicola Bay, FL shellstock oysters surviving irradiation at 0.0, 1.0 and 3.0 kGy over a three week dry storage time at 4-6°C.

In Figure 16, results for the survival of TX oysters are shown. It was determined that the populations behaved similarly, immediately after and 1 day after irradiation. However, the 3.0 kGy group became significantly different from the control and 1.0 kGy groups 2 days after irradiation, as was seen with the FL oysters. The 1.0 kGy oysters became significantly different from the control group on day 4 after irradiation as detected by a Chi-Square test at the 99% confidence level. The irradiated TX oysters also continued dying at a rapid pace and likewise by day 14 no 3.0 kGy oysters were left. Also, day 22 was the day on

which the last 1.0 kGy oysters died and it this point only 31% of the nonirradiated oysters had died.

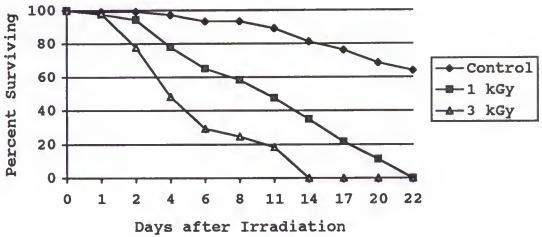

Figure 16. The percentage of Galveston Bay, TX shellstock oysters surviving irradiation at 0.0, 1.0 and 3.0 kGy over a three week dry storage time at 4-6°C.

Figure 17 was constructed as a combination of Figures 15 and 16, showing all shelf life data associated with both the FL and TX oysters. This figure shows just how similar the FL and TX oysters behaved at each of the doses. Perhaps, the most noticeable feature is that the control and 1.0 death curves are nearly identical. The 3.0 kGy group is also similar, but the initial death rate in the FL oysters is a bit higher. Notably, the day when the last oyster died was identical for both the FL and TX oysters. The control oysters had only experienced a 31 and 36% reduction in live numbers, respectively. The shelf life data can also be manipulated to yield D_{20} and D_{50} values.

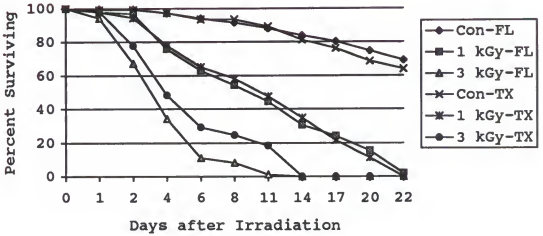

Figure 17. The percentage of Apalachicola Bay, FL and Galveston Bay, TX shellstock oysters surviving irradiation at 0.0, 1.0 and 3.0 kGy over a three week dry storage time at 4-6°C.

Table 3 was constructed as a quick reference as to the D_{20} and D_{50} values associated with irradiated and nonirradiated oysters.

Table 3. The D_{20} and D_{50} values of FL and TX irradiated and nonirradiated shellstock oysters.

	Control	1.0 kGy	3.0 kGy
Florida - D_{20}	17 days	4 days	2 days
Florida - D_{50}	> 25 days	9 days	3 days
Texas - D_{20}	14 days	4 days	2 days
Texas - D_{50}	> 25 days	11 days	4 days

The D_{50} values are extremely important in that the D_{50} is comparable to a lethal dose fifty, and one can assume that once 50% of the oysters have died, the other half will soon follow. The D_{20} is of extreme importance to oyster processors and sellers in that a 20% loss can have

serious economic implications. This means that irradiation may not have merit in oyster irradiation, no matter how clean the oysters are, if there is a 20% product loss before all of the product can be sold.

Shell to Meat Ratio

The shell to meat ratio was calculated for the FL and TX shellstock oysters used in the irradiation study and the shell to meat ratio of LA oysters was determined on a separate occasion. This ratio is important because there are differences in shell densities and meat sizes across these three geographical locations. An oyster shell with a higher density may actually attenuate the radiation dose delivered, and thus, "thicker" shells may need a higher dose of irradiation for the same effect obtained in "thinner" shells. Table 4 shows the shell to meat ratios calculated two ways: 1.) by adding up all of the shell weights, and dividing by the total amount of meat weights and 2.) to calculate a shell to meat ratio for each oyster, and then calculate an average of all of those. The "average" shell to meat ratio calculated, exceeded those that "total" ratios in all three locations.

It was determined that FL oysters had the smallest shell to meat ratios with a range of 4.81 to 5.07. The next largest ratios were detected in the TX oysters which had a range of 5.51 to 5.80. Finally, the LA oysters had the greatest shell to meat ratio with about a 7.16 to

7.68 range. This corresponds quite well with what can be seen by the naked eye -- LA oysters are considerably larger than TX or FL oysters.

Table 4. Shell to meat ratios for FL, TX and LA shellstock oysters.

State and Number of oysters	Total Shell Weight (grams)	Total Meat Weight (grams)	Shell to Meat Ratio (range)	Average of all S/M Ratios
FLORIDA (100)	11383	2367	4.81 (3.16-9.54)	5.07 (±1.02)
TEXAS (87)	7609	1380	5.51 (3.19-10.54)	5.80 (±1.32)
LOUSIANA (92)	9792	1367	7.16 (3.64-22.3)	7.68 (±1.73)

Identification of Surviving Organisms

Another critical feature of this irradiation experiment was to identify any organisms that survived irradiation. Isolated colonies were tested on the API 20e™ Biochemical Identification of Enterobacteriaceae. All of the organisms tested were either isolated on PCA, PCAS, TCBS, CPC or EC-MUG after 0, 1.0 or 3.0 kGy of exposure. Table 5. shows the organisms identified in Apalachicola Bay, FL oysters post-irradiation. This table identifies the actual dose from which the organism was selected, the name of the organism, the identification status, as well as the actual media from which it was isolated. In general, there were many different Vibrio spp. identified, as well as some other bacteria common to oysters such as Aeromonas spp. and

Citrobacter spp. The most notable organism to be identified in the FL oysters was the Salmonella spp.

Table 5. The biochemical identification of bacteria isolated from Apalachicola Bay, FL shellstock oysters irradiated at 0.0, 1.0 and 3.0 kGy using the API 20e™ Identification System of Enterobacteriaceae.

Treatment Group	Organism Identified	Identification Status	Media Isolated From
Control	Aeromonas hydrophila	Very Good ID	PCAS
	Citrobacter freundii	Excellent ID	PCAS
	Escherichia coli	Excellent ID	EC-MUG
	Klebsiella pneumoniae	Excellent ID	PCA
	Salmonella spp.	Excellent ID	PCA
	Serratia odorifera	Excellent ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio fluvialis	Excellent ID	PCAS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC
1.0 kGy	Aeromonas hydrophila	Very Good ID	PCAS
	Citrobacter freundii	Excellent ID	PCAS
	Escherichia coli	Excellent ID	EC-MUG
	Salmonella spp.	Excellent ID	PCA
	Serratia odorifera	Excellent ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio fluvialis	Excellent ID	PCAS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC
3.0 kGy	Aeromonas hydrophila	Very Good ID	PCAS
	Enterobacter cloacae	Excellent ID	PCA
	Escherichia coli	Excellent ID	EC-MUG
	Pseudomonas pseudomallei	Very Good ID	PCA
	Serratia liquefaciens	Very Good ID	PCA
	Serratia marcescens	Excellent ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC

This is important to note that the detection of a single Salmonella positive sample is enough to close that harvest area. Perhaps the most noteworthy piece of data

is that V. vulnificus were still detected even at doses as high as 3.0 kGy. This is significant because V. vulnificus has been previously characterized by Dixon (1992) as quite radiosensitive in phosphate buffered saline. However, in the oyster, it appears that V. vulnificus may be given a protective effect against irradiation either by the meat itself, or by the oyster shell. Furthermore, some other organisms to be identified that are of interest were the Klebsiella pneumoniae and a Serratia odorifera in the nonirradiated oysters. In the 3.0 kGy exposure, several new organisms were identified, Enterobacter cloacae, Pseudomonas pseudomallei, and two others, Serratia marcescens and Serratia liquefaciens that were not identified at 0.0 or 1.0 kGy. Perhaps some other species of bacteria inhibited these bacteria before irradiation at 3.0 kGy and thus these resistant oragnisms were then able to grow and divide without competition from others after irradiation.

Table 6 shows the organisms identified in the Galveston Bay, TX oysters post-irradiation. Again, there were a variety of Vibrio spp. identified at all three doses. Furthermore, there was a Salmonella spp. colony identified in these Texas oysters as well. There is a zero tolerance associated with the presence of any Salmonella spp. in oysters and these oysters were also positive for Salmonella spp.

Table 6. The biochemical identification of bacteria isolated from Galveston Bay, TX shellstock oysters irradiated at 0.0, 1.0 and 3.0 kGy using the API 20e™ Identification System of Enterobacteriaceae.

Treatment Group	Organism Identified	Identification Status	Media Isolated From
Control	Aeromonas hydrophila	Very Good ID	PCAS
	Citrobacter freundii	Excellent ID	PCAS
	Escherichia coli	Excellent ID	EC-MUG
	Proteus vulgaris	Excellent ID	PCA
	Salmonella spp.	Excellent ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio fluvialis	Excellent ID	PCAS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC
1.0 kGy	Aeromonas hydrophila	Very Good ID	PCAS
	Citrobacter freundii	Excellent ID	PCAS
	Escherichia coli	Excellent ID	EC-MUG
	Proteus vulgaris	Excellent ID	PCA
	Salmonella spp.	Excellent ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio fluvialis	Excellent ID	PCAS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC
3.0 kGy	Aeromonas hydrophila	Very Good ID	PCAS
	Citrobacter freundii	Excellent ID	PCAS
	Enterobacter gergoviae	Excellent ID	PCA
	Escherichia coli	Excellent ID	EC-MUG
	Pseudomonas pseudomallei	Very Good ID	PCA
	Vibrio alginolyticus	Excellent ID	TCBS
	Vibrio parahaemolyticus	Excellent ID	TCBS
	Vibrio vulnificus	Excellent ID	TCBS/CPC

The other organisms identified consisted again of Aeromonas spp. and Citrobacter spp. with an Enterobacter gergoviae and Pseudomonas pseudomallei organism identified at only the 3.0 kGy dose. Perhaps some other of bacteria were inhibiting these two before irradiation at 3.0 kGy, and thus these two resistant organisms were

then able to grow and divide without competition from others after irradiation.

Internal Dosimetry of Shellstock Oysters

Data from this experimentation was obtained only on a 4 dosimeter strips, but the data was clear. The dosimeters used inside of the oysters exposed to 1.0 kGy recorded a dose of only 0.5 kGy, while the dosimeters in the 3.0 kGy oysters only recorded 1.6 kGy. This is critical because the internal meat structure is not receiving the same dose that the dosimeters outside of the box are recording. Thus, bacterial destruction will not as great.

Determination of V. vulnificus D_{10} Value in ASW

This phase of the experimentation consisted of determining the D_{10} values for logarithmic phase, stationary phase, and VBNC forms of V. vulnificus. These values are critical in determining the radiation dose necessary for control of this bacteria, depending on growth phase or nonculturability and growth media.

Stationary phase (O, T and B) D_{10} Value in ASW

The D_{10} values of stationary phase V. vulnificus cells are depicted in Figures 18-20. These figures show the linear regression analysis (best fit straight line) of the irradiation of stationary phase V. vulnificus. The R^2 and equation of the line are shown for the CPC agar only, as well as the D_{10} calculation. CPC agar was selected because of its high selectivity for V.

vulnificus compared to the other media. In Figure 18, the D_{10} value associated with the O morphotype in ASW was determined to be D_{10} = 0.055 kGy on TCBS agar, D_{10} = 0.059 kGy on CPC agar and D_{10} = 0.057 kGy on APA agars. These values coincide quite closely with Dixon (1992) who found the D_{10} = 0.062 kGy for an 18-24 hour O V. vulnificus in phosphate buffered saline. The R^2 was found to be 0.981 and the equation of the line was calculated as y = -17.04x + 122.5 on the CPC agar.

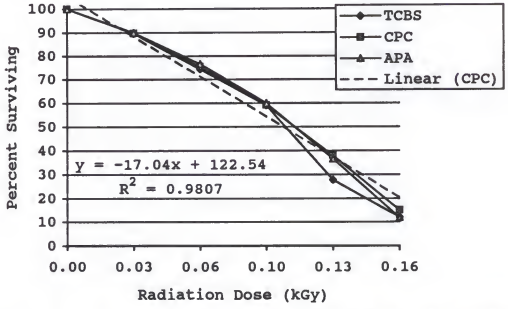

Figure 18. Plot of surviving fraction versus radiation dose for stationary phase "O" Vibrio vulnificus C7184 in ASW, plated on TCBS (D_{10} = 0.055 kGy), CPC (D_{10} = 0.059 kGy) and APA (D_{10} = 0.057 kGy) agars.

In Figure 19, the D_{10} value associated with the "blue-bug" or mutant, which is an O morphotype, in ASW was determined to be D_{10} = 0.056 kGy on TCBS agar, D_{10} = 0.057 kGy on CPC agar and D_{10} = 0.057 kGy on APA agar and D_{10} = 0.057 kGy on TN agar. These values again coincide

with Dixon (1992) who found the D_{10} = 0.062 kGy for an 18-24 hour O V. vulnificus in phosphate buffered saline. The R^2 was found to be 0.977 and the equation of the line was calculated as y = -17.52x + 123.3 on the CPC agar.

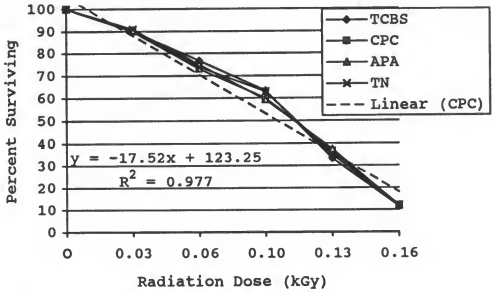

Figure 19. Plot of surviving fraction versus radiation dose for stationary phase "B" Vibrio vulnificus CVD 713 in ASW, plated on TCBS (D_{10} = 0.056 kGy), CPC (D_{10} = 0.057 kGy), APA (D_{10} = 0.057 kGy) and TN (D_{10} = 0.057 kGy) agars.

In Figure 20, the D_{10} value associated with the T morphotype in ASW was determined to be D_{10} = 0.045 kGy on TCBS agar, D_{10} = 0.043 kGy on CPC agar and D_{10} = 0.044 kGy on APA agars. These values are just slightly higher than those reported by Dixon (1992) who found the D_{10} = 0.037 kGy for an 18-24 hour T V. vulnificus in phosphate buffered saline. The R^2 was found to be 0.913 and the equation of the line was calculated as y = -23.26x + 131.1 on the CPC agar.

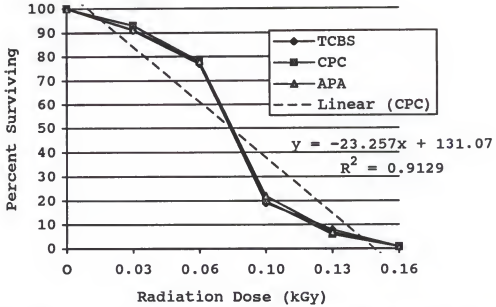

Figure 20. Plot of surviving fraction versus radiation dose for stationary phase "T" Vibrio vulnificus C7184 in ASW, plated on TCBS (D_{10} = 0.045 kGy), CPC (D_{10} = 0.043 kGy) and APA (D_{10} = 0.044 kGy) agars.

Logarithmic phase (O, T and B) D_{10} Value in ASW

The D_{10} values of logarithmic phase V. vulnificus cells are depicted in Figures 21-23. These figures show the linear regression analysis (best fit straight line) of the irradiation of all forms of V. vulnificus. The R^2 and equation of the line are shown for the CPC agar only, as well as the D_{10} calculation. CPC agar was selected because of its high selectivity for V. vulnificus compared to the other media. In Figure 21, the D_{10} value associated with the O morphotype in ASW was determined to be D_{10} = 0.054 kGy on TCBS agar, D_{10} = 0.053 kGy on CPC agar and D_{10} = 0.053 kGy on APA agars. These values coincide quite closely with Dixon (1992) who found the D_{10} = 0.062 kGy for an 18-24 hour O V. vulnificus in

phosphate buffered saline. The R^2 was found to be 0.993 and the equation of the line was calculated as y = -18.86x + 123.2 on the CPC agar.

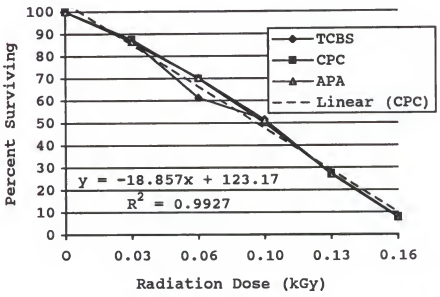

Figure 21. Plot of surviving fraction versus radiation dose for log phase "O" Vibrio vulnificus C7184 in ASW, plated on TCBS (D_{10} = 0.054 kGy), CPC (D_{10} = 0.053 kGy) and APA (D_{10} = 0.053 kGy) agars.

In Figure 22, the D_{10} value associated with the "blue-bug" in ASW was determined to be D_{10} = 0.053 kGy on TCBS agar, D_{10} = 0.054 kGy on CPC agar, D_{10} = 0.053 kGy on APA agar and D_{10} = 0.054 kGy on TN agar. These values again coincide with Dixon (1992) who found the D_{10} = 0.062 kGy for 18-24 hour O V. vulnificus in phosphate buffered saline. The R^2 was found to be 0.995 and the equation of the line was calculated as y = -18.69x + 121.9 on the CPC agar.

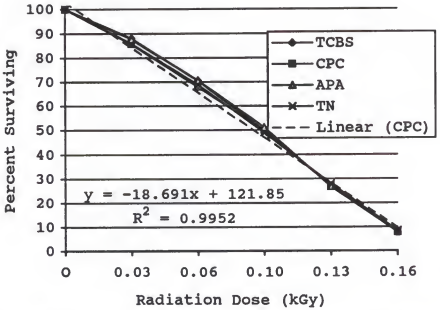

Figure 22. Plot of surviving fraction versus radiation dose for log phase "B" Vibrio vulnificus CVD 713 in ASW, plated on TCBS (D_{10} = 0.053 kGy), CPC (D_{10} = 0.054 kGy), APA (D_{10} = 0.053 kGy) and TN (D_{10} = 0.054 kGy) agars.

In Figure 23, the D_{10} value associated with the T morphotype in ASW was determined to be D_{10} = 0.043 kGy on TCBS agar, D_{10} = 0.043 kGy on CPC agar and D_{10} = 0.043 kGy on APA agars. These values are just slightly higher than those reported by Dixon (1992) who found the D_{10} = 0.037 kGy for an 18-24 hour T V. vulnificus in phosphate buffered saline. The R^2 was found to be 0.909 and the equation of the line was calculated as y = -23.17x + 129.3 on the CPC agar.

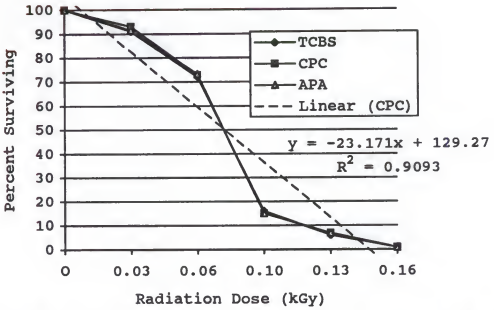

Figure 23. Plot of surviving fraction versus radiation dose for log phase "T" Vibrio vulnificus C7184 in ASW, plated on TCBS (D_{10} = 0.043 kGy), CPC (D_{10} = 0.043 kGy) and APA (D_{10} = 0.043 kGy) agars.

VBNC (O, T and B) D_{10} Value in ASW

The D_{10} values of VBNC V. vulnificus cells (O, T and B) are depicted in Figures 24-26. These figures show the linear regression analysis (best fit straight line) of the irradiation of VBNC V. vulnificus. The R^2 and equation of the line are shown for the DVC only, as well as the D_{10} calculation. This is presented first, because immediately after irradiation, the only way to count VBNC cells is by the direct viable count, otherwise a 24-48 hour resuscitation is required before growth can be detected on microbiological media. In Figure 24, the D_{10} value of the VBNC O morphotype cells was determined to be 0.165 kGy. The equation of the line generated from the death curve is y = -6.06x + 107.76, with a corresponding

R^2 value of 0.979. This D_{10} value is 3 times larger than what was observed with the normal cells irradiated in ASW, indicating that the VBNC state provides increased radioresistance for the organism.

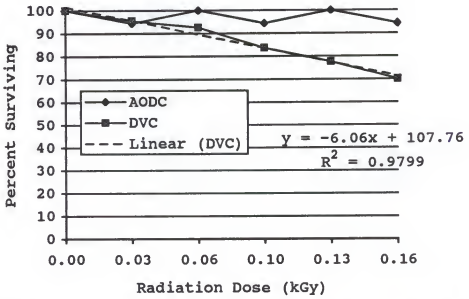

Figure 24. Plot of surviving fraction versus radiation dose for VBNC "O" Vibrio vulnificus C7184 in ASW, as detected by DVC (D_{10} = 0.165 kGy) and AODC.

In Figure 25, the D_{10} value of the VBNC "blue-bug" (B), which is an O morphotype cell, was determined to be 0.173 kGy, which is very close to the 0.165 kGy observed with the O morphotype in Figure 24. The equation of the line generated from the death curve is y = -5.75x + 108.47, with a corresponding R^2 value of 0.957. This D_{10} value is more than 3 times larger than what was observed with the normal cells irradiated in ASW, indicating again that the VBNC state provides increased radioresistance for the organism.

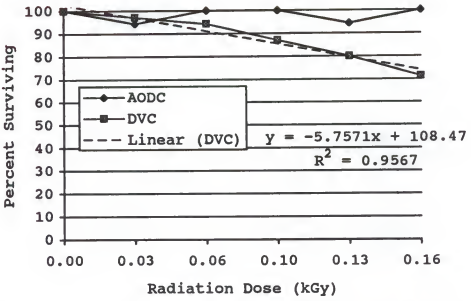

Figure 25. Plot of surviving fraction versus radiation dose for VBNC "B" Vibrio vulnificus CVD 713 in ASW, as detected by DVC (D_{10} = 0.173 kGy) and AODC.

The trend of increased radioresistance in the VBNC cells is continued with the T morphotype. In Figure 26, the D_{10} value of the VBNC T morphotype cells was determined to be 0.147 kGy. The equation of the line generated from the death curve is y = -6.83x + 107.78, with a corresponding R^2 value of 0.988. This D_{10} value is 3 times larger than what was observed with the normal cells irradiated in ASW, indicating that even with the nonencapsulated cells, the mechanisms that lead to nonculturability, also help provides increased radioresistance for the organism.

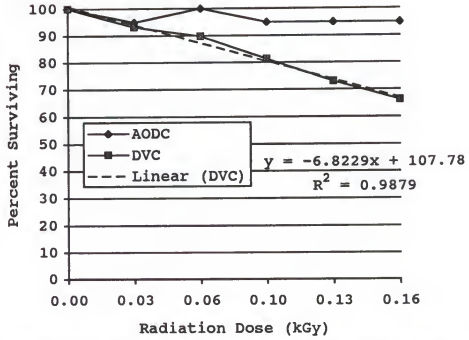

Figure 26. Plot of surviving fraction versus radiation dose for VBNC "T" Vibrio vulnificus C7184 in ASW, as detected by DVC (D_{10} = 0.147 kGy) and AODC.

Resuscitation of VBNC Post-Irradiation

Samples of viable but nonculturable cells of V. vulnificus that were irradiated at different doses were also incubated at room temperature for 24 and 48 hours and checked for resuscitation post-irradiation. Figures 27-32 depict the resuscitation profiles of irradiated O, T and B VBNC V. vulnificus cells. In Figure 27, 24 hours after irradiation, culturability begins to return close to the DVC values of nearly 1 x 10^5 in the HIS and APA plates for the O C7184 strain, however the more selective TCBS and CPC plates are about 0.5 logs lower. This is seen at all doses, including zero dose.

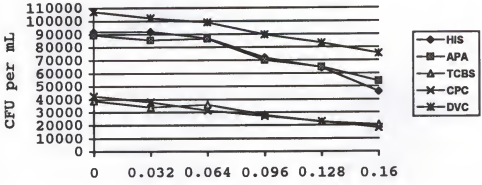

Figure 27. Room temperature (25°C) resuscitation of irradiated VBNC "O" Vibrio vulnificus in ASW after 24 hours and plating on HIS, TCBS, CPC, APA and a DVC.

In Figure 28, it is shown that culturability begins to return close to the DVC values of nearly 1 x 10^5 in the HIS and APA plates for the mutant B CVD 713 after 24 hours of room temperature incubation. However, the more selective TN, TCBS and CPC plates are still about 0.5 logs lower. This is again seen at all doses including zero dose.

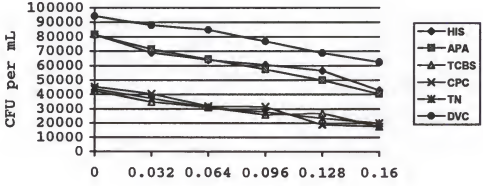

Figure 28. Room temperature (25°C) resuscitation of irradiated VBNC "B" Vibrio vulnificus in ASW after 24 hours and plating on HIS, TCBS, CPC, TN, APA and a DVC.

In Figure 29, 24 hours after irradiation, culturability begins to return close to the DVC values of nearly 1 x 10^5 in the HIS and APA plates for the T C7184 strain, however the more selective TCBS and CPC plates are also about 0.5 logs lower. This trend is observed at all doses.

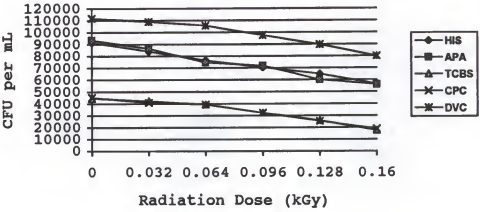

Figure 29. Room temperature (25°C) resuscitation of irradiated VBNC "T" Vibrio vulnificus in ASW after 24 hours and plating on HIS, TCBS, CPC, APA and a DVC.

Data in Figure 30 show that culturability returns to the direct viable count values for the O C7184 strain of over 1 x 10^5 in the HIS and APA plates, as well as the more selective TCBS and CPC plates, 48 hours after irradiation. The numbers obtained on all media are nearly equal to the DVC indicating that complete resuscitation has occurred. It should be noted that the plate counts never exceeded the DVC showing that resuscitation of VBNC cells occurred and not the growth of a few culturable cells.

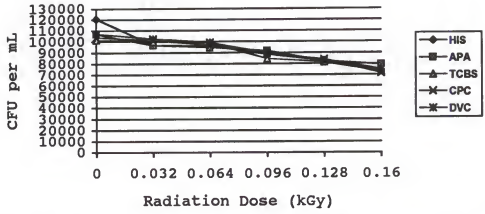

Figure 30. Room temperature (25°C) resuscitation of irradiated VBNC "O" Vibrio vulnificus in ASW after 48 hours and plating on HIS, TCBS, CPC, APA and a DVC.

In Figure 31, the data show that culturability also returns to the levels of the direct viable count values for the B CVD 713 strain of over 1 x 10^5 on the HIS, APA, TN, TCBS and CPC plates, 48 hours after irradiation,.

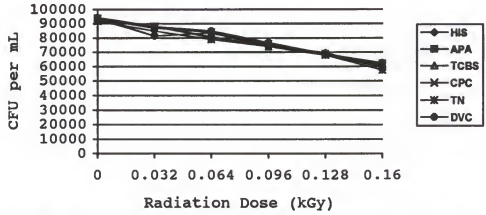

Figure 31. Room temperature (25°C) resuscitation of irradiated VBNC "B" Vibrio vulnificus in ASW after 48 hours and plating on HIS, TCBS, CPC, TN, APA and a DVC.

The numbers obtained on all media are again nearly equal to the DVC indicating that complete resuscitation has occurred. Similarly, the plate counts never exceeded the

DVC showing that resuscitation of VBNC cells occurred, and not the growth of a few culturable cells.

In Figure 32, 48 hours after irradiation, culturability returns all the way up to the direct viable count values for the T C7184 strain of over 1 x 10^5 on HIS, APA, TCBS and CPC plates. The numbers again indicate resuscitation as they are nearly equal to the DVC, without ever exceeding the DVC showing that resuscitation of VBNC cells occurred, and not the growth of a few culturable cells.

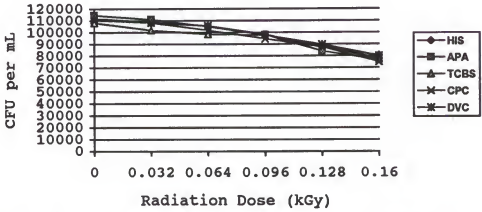

Figure 32. Room temperature (25°C) resuscitation of irradiated VBNC "T" Vibrio vulnificus in ASW after 48 hours and plating on HIS, TCBS, CPC, APA and a DVC.

There is a lot of criticism involving the resuscitation of cells. There is another school of thought claiming that true resuscitation does not really occur (except for maybe a few cells). Rather the increase in bacterial numbers seen on plating media is due to the growth and division of either a few cells that

have resuscitated, or a few cells that remained culturable all along.

To address this controversy, the easiest method to answer the question is by the addition of nalidixic acid to resuscitating cells. In this case, any culturable cells present in the sample would be inhibited by the nalidixic acid in terms of DNA replication and hence cell division. The nonculturable cells would then be the only organisms under consideration. If they truly were resuscitated, then once the 24-48 hours of room temperature resuscitation passed, they alone would be the source of the positive growth on HIS, APA, TN, TCBS or CPC agars.

A separate set of irradiation experiments were conducted to determine the effects of adding nalidixic acid to the resuscitation medium after irradiation. It was determined in the presence of nalidixic acid, irradiated VBNC cells (O, T and B) resuscitated at room temperature, for 48 hours, reached levels equal to the original value determined in the DVC. No graph is presented for this finding as the parameters for inducing the VBNC state and irradiation protocol were followed identically to the that listed previously, and thus all of the numbers were very similar to the numbers presented in Figures 27-32. The findings of this research indicate that true resuscitation occurred after the irradiation of

VBNC cells (O, T and B), and not the regrowth of culturable cells.

Other influences on resuscitation can be the microbial concentration in the sample, as well as the resuscitation medium. Two separate experiments were designed to address this issue. First, after the irradiation of VBNC cells (O, T and B), 2.0 mL undiluted samples were allowed to resuscitate at room temperature for 48 hours, as well as 10-, 100- and 1000-fold dilutions. The undiluted samples resuscitated back to the original DVC values, but none of the diluted samples resuscitated.

The effects of resuspension media were also assessed. A whole 750 mL microcosm was spun down, yielding a pellet. This pellet was washed 2X and subsequently resuspended in fresh ASW. The result was complete resuscitation as was seen before. However, when this fresh resuspension was diluted 10-, 100- and 1000-fold, no resucitation occurred. The first possibility is that the ASW has nothing to do with the resuscitation, as resuscitation occurred in both the fresh and old ASW. However, there is some concentration effect, since diluted samples of the VBNC microcosms did not resuscitate. Perhaps the organisms must be present in a specific concentration so that they can produce some factor which promotes resuscitation. Perhaps there are compounds already present in the microcosm, and they are

captured along with the cells during centrifugation. If so, compounds could be transferred with the cells to new seawater. A dilution of these cells and compounds may result in levels that are not usable by the bacteria. It seems that the cells are not as dependent on the ASW environment as they are on each other.

SUMMARY AND CONCLUSIONS

One of the primary objectives of this research was to determine the effects of ionizing radiation on FL and TX oysters on a true commercial scale in terms of shelf life (D_{20} and D_{50} values) and the microbiological consequences. Furthermore, these shellstock oysters were evaluated in terms of shell to meat ratio, internal shell dosimetry and the identification of any organisms surviving irradiation for up to 14 days in dry cold storage. The other aspect of the research dealt with determining the D_{10} values of the logarithmic and stationary phase of V. vulnificus strains C7184 (O, T) and mutant CVD 713 (B) in ASW and on a variety of media The VBNC form of these three V. vulnificus forms was also induced and the subsequent D_{10} values calculated for the VBNC cells using the direct viable count. In addition, the effects of radiation on 24 and 48 hour room temperature resuscitation was monitored on a variety of solid media and compared to the corresponding DVC. Also, an assessment was made as to whether or not true resuscitation occurred or whether the growback of few remaining culturable cells occurred. Furthermore, the effects of the type of media used for resuscitation (old

or fresh ASW) was evaluated, as was the effect of different microbial concentrations on resuscitation.

In terms of shelf life, this research shows that the D_{20} values for FL shellstock oysters were 17, 4 and 2 days for the 0.0, 1.0 and 3.0 kGy exposures, respectively. The D_{20} values for TX shellstock oysters were 14, 4 and 2 days for the 0.0, 1.0 and 3.0 kGy exposures, respectively. The D_{50} values for FL shellstock oysters were >25, 9 and 3 days for the 0.0, 1.0 and 3.0 kGy exposures, respectively, whereas the D_{50} values for TX shellstock oysters were >25, 11 and 4 days for the 0.0, 1.0 and 3.0 kGy exposures, respectively. It is apparent that there is a serious decrease in shelf life associated with the irradiated oysters and that a serious product loss can be expected with irradiating summer oysters.

However, if the microbial levels are reduced to undetectable or very low levels, then the benefit of clean oysters may outweigh the rapid product loss. However, this is not the case. Immediately following irradiation, there is a decrease of about 2 logs in total bacterial numbers and Vibrio levels regardless of source or dose. However, this lower value is only maintained for a few days and then the bacterial numbers begin to rise back up to the nonirradiated, control values or even higher. The oysters are dying rapidly and the bacteria (total plate count, Vibrios and fecals) are multiplying

rapidly. There are then very few days allowed for transport, stoarge, sale and consumption of irradiated oysters before they become unfit or die. This research also revealed the surviving organisms post-irradiation to be genera such as Vibrio spp., E. coli, Aeromonas and a few spoilage organisms such as Pseudomonas and Serratia. Furthermore, it was determined that the shell to meat ratios of FL, TX and LA oysters were 4.8, 5.5 and 7.2, respectively. Perhaps the most interesting piece of data surrounded the determination of the internal doses recieved inside the shell itself. The D_{max}/D_{min} ratios of 2:1 and 1.5:1 for the 1.0 kGy and 3.0 kGy exposures as detected by FTS, did not correlate well with the data obtained from the internal dosimters. It is quite clear that the internal part of the oyster received about half of the dose that the outside of the box received and that the density (shell to meat ratio is an indication) of the shellstock oysters is leading to true attenuation and thus a decreased dose. This, probably helps to explain the variability experienced when testing the oysters microbiologically as certain oysters are not getting the same internal dose and thus not the same bactericidal killing power. These "less-irradiated" oysters probably lead to the elevated counts sometimes experienced during dry storage sampling.

The other phase of experimentation dealt with the organism, V. vulnificus. The D_{10} values of (O, T and B)

V. vulnificus were determined both in the logarithmic and stationary phase in ASW and plated on a variety of media. The D_{10} values are displayed in Table 7.

Table 7. D_{10} values for log and stationary phase V. vulnificus on APA, TCBS, CPC and TN agars.

	APA	TCBS	CPC	TN
O Stationary	0.057	0.055	0.059	--
O Logarithmic	0.053	0.054	0.053	--
B Stationary	0.057	0.056	0.057	0.057
B Logarithmic	0.053	0.053	0.054	0.054
T Stationary	0.044	0.045	0.043	--
T Logarithmic	0.043	0.043	0.043	--

These values correspond quite well with Dixon (1992) with the O morphotype having a bit lower D_{10} in ASW than PBS and the T morphotype a bit higher in ASW than in PBS. As expected the mutant B type responded similarly to the the O morphotype, since in fact it is a genetically altered O type. No significant difference could be detected between the different types of media used in the growth after irradiation. Furthermore, there was no significant difference detected between the stationary and log phases. The D_{10} values were calculated for the VBNC form of these cells and they are displayed in Table 8. These D_{10} values for the VBNC cells are a full three times larger than what is observed for the normal, culturable cells. This indicates that the induction of

the VBNC mechanisms of survival help provide an increased radioresistance fo the organism. This is most likely accomplished in the bacteria by reductive division whereby there is a compacting of the nuclear region, DNA rearrangement and a lessening of DNA per cell (Brauns et al., 1991). If this is the case, the probability of a hit decreases with decreasing nucleus size. Furthermore, compacted DNA would be wound so tight that the backbone structure would have less area exposed for a hit of gamma rays.

Table 8. The D_{10} values determined for VBNC cells of V. vulnificus using the direct viable count.

	DVC
O VBNC	0.165
B VBNC	0.173
T VBNC	0.147

In addition to the increased resistance of the VBNC cells of V. vulnificus, the resuscitation patterns after irradiation were examined. It was determined that complete resuscitation occurred after 48 hours of room temperature incubation, with levels matching the original direct viable count, but not higher. Resuscitation was guaranteed in this case by the addition nalidixic acid to the resuscitation medium, which prevented the growth of any culturable cells that may have been present in the

sample. The plate counts after 48 hours at room temperature were again equal to the DVC, but not higher.

The type of resuscitation media and microbial concentration during resuscitation were also examined. After the irradiation of VBNC cells (O, T and B), 2.0 mL undiluted samples were allowed to resuscitate at room temperature for 48 hours, as well as 10-, 100- and 1000-fold dilutions. The undiluted samples resuscitated to the original DVC values, but none of the diluted samples resuscitated. Full microcosms of VBNC cells were harvested by centrifugation, washed 2X, and resuspended for resuscitation in fresh ASW. It was determined that complete resuscitation occurred in the fresh seawater. However, when diluting the fresh ASW 10-, 100- and 1000-fold, no resuscitation occurred. This indicates that the resuscitation media (old ASW) is not harboring vital nutrients that induce resuscitation, because new ASW allows for resuscitation. The focus is probably properly placed on the actual cell concentration, as there appears to be a cooperative effect among the cells as concentrations below 10^5 per mL would not resuscitate.

In conclusion, irradiation processing cannot be considered as a method to sterilize shellstock oysters, and provide a shelf stable product. Irradiation can reduce some pathogens and viruses, perhaps below their infective dose, but not rid the shellstock oyster completely of all contaminants. The shellstock oyster

poses many challenges to irradiation and food processing technology. These problems include uneven dose distribution, different shell to meat ratios for oysters from different geographic locations and the potential for growback of organisms in the irradiated product over time. Furthermore, oysters are lives animals with their own inherent radiation sensitivity, and thus radiation D value. It is clear that the D_{10} value of oysters falls somewhere in the range where bacterial and viral reduction is observed. Thus, the conundrum of deciding on the dose that will give the best shelf life and maximum bacterial reduction will continue. The survival of organisms is a great concern because when competition is altered between the flora, the result could be the rapid outgrowth of a potentially dangerous microbe.

Concerning the irradiation of V. vulnificus, it is apparent that in simple media like ASW, it is a very radiosensitive microorganism. However, in a complex system like a shellstock oysters, there is a protective effect by the shell itself and this organism can survive. More importantly it can grow and divide in dry cold storage, or even worse enter the viable but nonculturable state. The VBNC forms of V. vulnificus are 3X more resistant to radiation than the corresponding culturable forms and this too could be a potential problem in winter harvest oysters that may have VBNC cells. There is evidence from this research for the presence of

resuscitation of VBNC *V. vulnificus* cells post-irradiation and that cell concentration plays a key role in the resuscitation process.

APPENDIX

MICROBIOLOGICAL CONSEQUENCES

Table A. The Microbiological Analysis of FL (A0, A1, A3) and TX (T0, T1 and T3) Shellstock Oysters Irradiated at 0, 1.0 and 3.0 kGy and then Kept Under Dry Storage at 4-6°C Over a Two Week Time Period.

Day 0	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	10,900	16,500	23,000	23,000	23,000	210	210
A1	2050	950	4900	4900	4900	330	330
A3	30	85	2300	2300	2300	63	0
T0	18,500	18,000	23,000	23,000	23,000	230	230
T1	15	65	4900	4900	4900	200	200
T3	30	30	2300	2300	2300	0	0

Day 2	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	11,900	11,400	49,000	49,000	49,000	490	490
A1	8050	9,400	7900	7900	7900	130	130
A3	1550	305	4900	4900	4900	45	0
T0	15,600	29,000	49,000	49,000	49,000	490	490
T1	2500	3950	23,000	23,000	23,000	490	490
T3	380	1330	7900	7900	7900	490	490

Day 4	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	68,000	185,000	230,000	49,000	33,000	2300	2300
A1	7050	11,400	23,000	13,000	13,000	1300	1300
A3	6550	5100	23,000	13,000	13,000	1300	130
T0	23,500	39,000	230,000	49,000	49,000	1300	1300
T1	3300	5250	23,000	13,000	13,000	790	790
T3	505	1830	23,000	2000	2000	230	130

Day 7	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	50,500	230,000	490,000	49,000	49,000	2300	2300
A1	5000	3300	33,000	13,000	13,000	230	130
A3	5500	5650	23,000	13,000	13,000	490	490
T0	181,000	201,000	490,000	230,000	130,000	2300	2300
T1	165,000	157,000	230,000	230,000	23,000	330	230
T3	31,000	49,000	23,000	23,000	23,000	230	230

PCA= plate count agar, PCAS = plate count agar + 2.5% NaCl, MPN = alkaline peptone water most probable number, TCBS = thiosulfate citrate bile salts sucrose agar, CPC= colistin polymixin cellobiose agar, LB = Lactose Broth and EC-MUG = *E. coli* broth with MUG

Table A.--continued.

Day 9	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	60,000	237,000	490,000	49,000	49,000	230	230
A1	22,500	174,000	49,000	49,000	33,000	330	130
A3	25,000	239,000	49,000	49,000	33,000	230	230
T0	220,000	255,000	230,000	230,000	230,000	230	230
T1	95,000	345,000	130,000	130,000	73,000	230	230
T3	14,500	16,100	23,000	23,000	23,000	230	230

Day 11	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	54,000	207,000	490,000	73,000	73,000	330	330
A1	14,500	177,000	49,000	49,000	49,000	230	230
A3	21,000	177,000	23,000	23,000	23,000	230	130
T0	265,000	190,000	110,000	110,000	110,000	330	330
T1	134,000	220,000	70,000	70,000	70,000	230	130
T3	17,000	21,500	23,000	23,000	23,000	230	130

Day 14	PCA	PCAS	MPN	TCBS	CPC	LB	EC-MUG
A0	63,000	190,000	490,000	230,000	230,000	700	700
A1	24,000	177,000	230,000	230,000	230,000	330	330
A3	15,000	136,000	49,000	49,000	23,000	230	230
T0	234,000	188,000	230,000	230,000	230,000	1400	1400
T1	165,000	162,000	170,000	170,000	170,000	330	130
T3	20,000	29,000	23,000	23,000	23,000	230	130

PCA= plate count agar, PCAS = plate count agar + 2.5% NaCl, MPN = alkaline peptone water most probable number, TCBS = thiosulfate citrate bile salts sucrose agar, CPC= colistin polymixin cellobiose agar, LB = Lactose Broth and EC-MUG = E. coli broth with MUG

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BIOGRAPHICAL SKETCH

Dustin William Dixon, the younger of Albert and Joyce Dixon's two children, was born July 6, 1968, in Tampa, Florida. He attended St. John's Parish Day School, and graduated with honors from Tampa Preparatory High School in 1986. He was awarded his Bachelor of Science in Agriculture (BSA) degree from the University of Florida in May, 1990, from the Department of Microbiology and Cell Science. He changed departments at the University of Florida for graduate study, to the Department of Food Science and Human Nutrition, in pursuit of his Master of Science degree under the supervision of Dr. Gary E. Rodrick. He was awarded the Master of Science degree in August of 1992. Furthermore, he was initiated into the honorary society of agriculture, Gamma Sigma Delta. He was awarded a .33 FTE assistantship to continue graduate study towards a Doctor of Philosophy degree in the Department of Food Science and Human Nutrition. He was awarded a Ph.D. in August of 1996, and plans to begin a career in either the food industry or academia.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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